Journal of Applied Botany and Food Quality 94, 92 - 98 (2021), DOI:10.5073/JABFQ.2021.094.011

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Morphology and phytochemistry of *Sanguisorba officinalis* L. seeds (Rosaceae)

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(Submitted: October 15, 2020; Accepted: April 29, 2021)

Summary

Great burnet (*Sanguisorba officinalis*) has been used as medicinal plant for more than 2000 years. However, little is known about the morphology and the secondary metabolites of its seeds. The investigations reported here focus on the morphology and the characterization of phenolics and fatty acids in *S. officinalis* seeds. For this purpose, dried seeds were investigated using scanning electron microscopy to clarify their compartment structures. Furthermore, the seeds were extracted with CH_2Cl_2 and MeOH to characterize the fatty acids and to assess the secondary metabolite profile. The seed structure consists of a floral cup, a brown pericarp with calcium oxalate crystals, a fibre layer and the seed kernel with its seed coat. Individual compounds were characterized by high performance liquid chromatography and gas chromatography coupled with mass spectrometric detection (HPLC-DAD-MS^n and GC/MS). CH_2Cl_2 extraction and GC investigations revealed the occurrence of fatty acids (29 % of the seed dry weight), with linoleic acid, linolenic acid, and oleic acid as major compounds. In addition, MeOH extracts were analyzed by LC/MS^n, which revealed the occurrence of flavonoids (quercetin, catechin, epicatechin), ellagitannins and caffeic acid derivatives.

Introduction

Members of the Rosaceae family are generally subdivided into three subfamilies: Rosoideae, Amygdaloideae, Dryadoideae. This family of flowering plants includes about 4,828 known species in 91 genera, which may grow as trees, shrubs, or herbaceous plants. This family is characterized by exceptional horticultural significance with economically important fruit-bearing plants. The fruits occur in many varieties and were once considered the main characters for the definition of the aforementioned subfamilies. The fruits are also characterized by great diversity and may occur as follicles, capsules, nuts, achenes, drupes and accessory fruits, like the pome of an apple, or the hip of a rose. Well-known examples of the three subfamilies are as follows: Amygdaloideae: plums, cherries, peaches, apricots, almonds, apples, pears, quince, ornamental shrubs such as *Exochorda*, *Sorbaria* and *Physocarpus*; Dryadoideae: *Cercocarpus*, *Chamaebatia*, *Dryas*, *Purshia*; Rosoideae: strawberries, blackberries and raspberries, roses and other ornamental and medicinal plants such as *Geum*, *Potentilla*, *Alchemilla* and *Sanguisorba* (Challice, 1974; McNeill, 2012; Simpson, 2018).

The genus *Sanguisorba* is comprised of approximately 18 to 34 species and subspecies, which are widely distributed in the Northern hemisphere of Eurasia and North America (GBIF Secretariat, 2019; Kurtto, 2009). The common name of *Sanguisorba* in western countries is burnet, and the plant is known to have hemostatic properties. The plants are perennial herbs with leaf rosettes, the stems of which grow 10 to 200 cm high, with further leaves arranged alternately up the stem. The leaves are pinnate with serrated margins. Flowers are small, tetramerous or trimerous and often unisexual, and they lack petals (Wang et al., 2020). The stamina have long filaments, and gynoecia consist of a single carpel topped with a feathery style (Kalkman, 2004). The flowers are small, dense clusters or spikes with a length of 1 to 7 cm (Blaschek et al., 2018; Uchida and Ohara, 2018). The flowering stage ranges from June to September and the fruit phases are usually from August to November. The fruits of *Sanguisorba* species belong to the nuts. The nuts of great burnet (*S. officinalis*) are narrowly winged (Fig. 1), with smooth surfaces, oval or broadly triangular in cross-section; not heteromorphic. The size ranges from 2.6 × 1.4 × 1.4 mm to 3.5 × 2.2 × 2.1 mm (Verband Botanischer Gärten, 2020). *S. officinalis* is a typical traditional Chinese medicinal plant. Especially the roots and herbal parts have been used to treat burns, hematemesis, asthma, intestinal infections and dermatitis (Lee et al., 2010; Yang et al., 2015; Yokozawa et al., 2002; Yu et al., 2011; Zhang et al., 2018). The primary biologically active constituents belong to the terpenoids, tannins and flavonoids, which are associated with antioxidant, anti-inflammatory, antiviral, antifungal, hemostatic and cytotoxic activities (Cai et al., 2012; Kim et al., 2008; Liang et al., 2013; Sun et al., 2012; Zhang et al., 2013; Zhang et al., 2012). There is an increasing interest of finding essential fatty oils needed for human health and well-being, for pharmaceuticals, nutritional foods or cosmetics. As an example, the seed oil of *Borago officinalis* L. is rich in gamma-linolenic acid and used as dietary food supplement and ingredient for cosmetic preparations (Asadi-Samani et al., 2014). Many traditional medicinal plants contain phenolic fatty oil in their seeds, which could be of interest for exactly these purposes. But there are only a few studies on almost forgotten medicinal plants and their primary and secondary metabolites of the seeds. Since studies on *S. officinalis* seeds are still rare, the present study aimed at a profound characterization of the phenolic compounds and reports the detailed structure of the seeds for the first time.

Materials and methods

Seeds of *Sanguisorba officinalis* L. (Year of harvest: 2015; Location: Germany; voucher number: HOH-022713; thousand-seed weight (TSW) = 2.04082 g), *Sanguisorba minor* Scop. (Year of harvest: unknown; Location: Germany; voucher number: HOH-022757; TSW = 8.33333 g), *Sanguisorba tenuifolia* Fisch. ex Link (Year of harvest: unknown; Location: Germany; voucher number: HOH-022755; TSW = 1.33333 g), *Sanguisorba parviflora* Takeda (*S. tenuifolia var. parviflora;* Year of harvest: unknown; Location: Germany; voucher number: HOH-022756; TSW = 1.0989 g) were acquired from *Jelitto Perenial Seeds GmbH* (Schwarmstedt, Germany). The species were identified by Dr. *R. Duque-Thüs* and voucher specimens were deposited with the Herbarium of the Institute of Botany, Hohenheim University.

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Dry seeds were cut with a razor blade and were photographed with a *Sony alpha 6000* (*SIGMA* 70 mm 1:2.8 DG MACRO + Macro lens). Seeds of *S. officinalis* were mounted on adhesive carbon tabs on aluminium stubs and sputter-coated with gold-palladium (20/80; 14-15 mA; 0.1 - 0.15 mbar; *SCD 040, Balzer Union, Wallruf,* Germany) and investigated using a scanning electron microscope *DSM 940* (*Zeiss*, Oberkochen, Germany) at 5 kV (Lorenz et al., 2018). Moreover, seeds of *S. officinalis* were photographed at different developmental stages, i.e. after pre-soak in water (5 days), after germination (8 days) and as seedling (14 days). Furthermore, seeds of *S. officinalis* (20.0 g) were immersed in CH_2Cl_2 (180 ml) and comminuted by *Ultra-Turrax* treatment (3 min; 21000 rpm, *IKA Werke GmbH & Co. KG*, Staufen, Germany), under external ice cooling. After maceration overnight at 4 °C the seeds were filtered off over *Celite* by vacuum suction and extracted a second time in the same manner. An oil fraction (5.74 g; 28.7% of the seed weight) was recovered from the combined CH_2Cl_2 extracts by vacuum rotoevaporation of the solvent. Subsequently, the degreased seeds were extracted twice with MeOH (180 ml) overnight (+ 4 °C), filtered off and MeOH was removed *in vacuo* to yield a crude extract. For GC analyses, fatty acid methyl esters were prepared by on-column derivatization with trimethylsulfonium hydroxide (TMSH, 0.25 M in MeOH). Briefly, 10 mg of viscous oil sample (CH_2Cl_2 residue) were dissolved in 2000 µl TBME. Aliquots of 10 µl of this test solution were mixed with 170 µl of TBME followed by 60 µl of TMSH. Subsequently, the mixture was directly injected into the GC/MS system (*PerkinElmer Clarus 500;* (Heinrich et al., 2017)).

For phenolic compound analysis, the previously mentioned MeOH crude extract (3-5 mg/ml) was redissolved in MeOH/H_2O (50/50, v/v). Liquid chromatographic analyses were carried out on *an Agilent 1200 HPLC* system (*Agilent Technologies*, Inc., Palo Alto, USA), equipped with a binary pump, a micro vacuum degasser, an autosampler, a thermostatic column compartment and a UV/VIS diode array detector. A *Kinetex*® C18 reversed-phase column (2.6 µm particle size, 150 × 2.1 mm i.d., *Phenomenex Ltd.,* Aschaffenburg, Germany) was used for chromatographic separation. The LC system was coupled to an *HCTultra* ion trap mass spectrometer (*Bruker Daltonik GmbH*, Bremen, Germany) with an ESI source operating in the negative ion mode (Bunse et al., 2020). For characterizing the compound profiles, three biological replicates (n = 3) of all samples were prepared. In addition, technical replicates were prepared to obtain the graphs, which allowed the calculation of standard deviations.

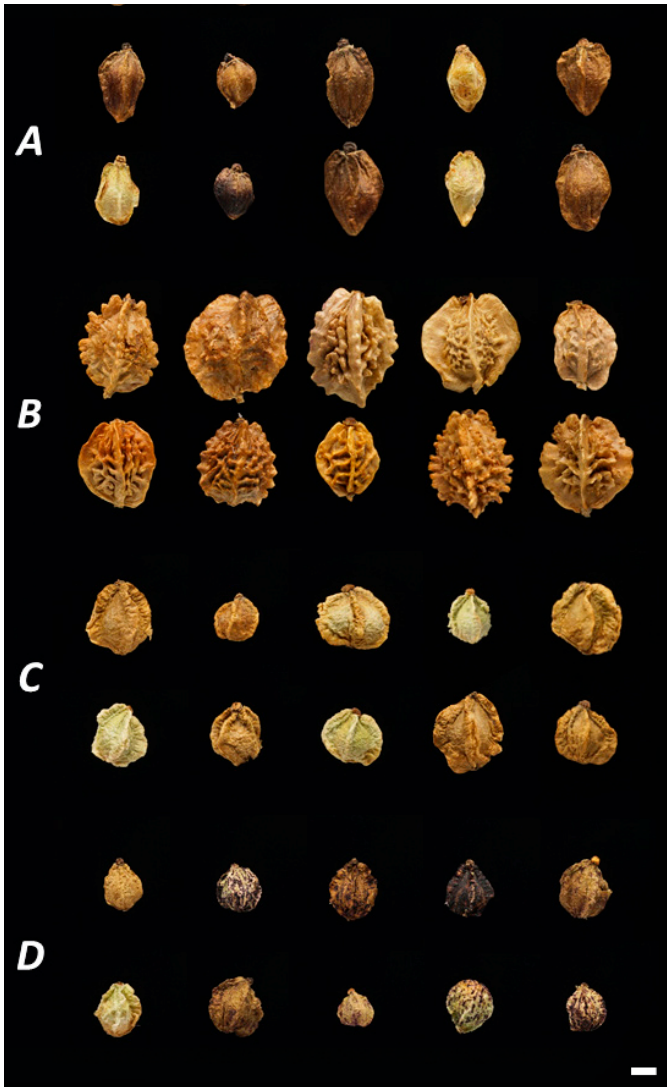

Fig. 1: Overview of seed shapes and their morphological variations of *S. officinalis* (A), *S. minor* (B), *S. tenuifolia* (C) and *S. parviflora* (D). Scale bare = 1 mm.

Results

Seed morphology

The nuts of *Sanguisorba* reveal species-dependent variations. Naturally, also nuts within a species reveal variations of their individual appearance (Fig. 1). Fig. 2 shows seeds and their cross sections of *S. officinalis* (A), *S. minor* (B), *S. tenuifolia* (C), and *S. parviflora* (D). The species A, C, D are characterized by similar narrowly winged seed shapes and contained only one seed kernel (achene), whereas the nuts of *S. minor* (B) usually contained 1-3 seed kernels per nut, and the hypanthium showed serrated netting strips.

For a more detailed investigation of the seed morphology, SEM images of the seed structures of *S. officinalis* were taken. The dried fruit (seed) of *S. officinalis* consists of several layers (Fig. 3). The floral cup or hypanthium forms the outer layer, the surface of the fruit. The pericarp is located below the perigynous hypanthium and encloses the inner seed kernel. The pericarp shows an outer layer with cells containing crystal structures. The cells located inside show a fibrous structure with thickened inner walls and extended cells. The pericarp converges at the apex of the seed and forms the stylus, which ends with the stigma in the flowering stage. The seed kernel is located inside the pericarp. It consists of the embryo with its two cotyledons, the epicotyl, the hypocotyl and the radicle covered by the seed coat.

Beside the aforementioned morphological structures, the longitudinal section of pre-swollen seeds (5 days) of great burnet (Fig. 4, A) showed the white endosperm of the cotyledons. Furthermore, SEM analyses revealed the epicotyl (e) of the embryo with the base of plumule. Fig. 4 B shows the primary root of a seedling 8 days after germination with a piece of seed coat at its tip. After 6 further days, a seedling of *S. officinalis* (Fig. 4, C) has developed a root (r), an elongated hypocotyl (h), green cotyledons (co), and the epicotyl shows primary leaves (pl, leaf bud) at an early developmental stage.

Lipid constituents

For investigating the lipid constituents, a CH_2Cl_2 extract from dried seeds of *S. officinalis* was prepared to yield a fatty oil (29% of dry weight of the seeds). GC/MS analyses revealed a complex peak profile. By comparing the mass spectra of individual components with those of reference compounds, a number of fatty acids with carbon-chain lengths of C_{16} - C_{22} were assigned (Fig. 5). Unsaturated fatty acids: 39% linolenic acid (C18:3), 36% linoleic acid (C18:2), 17%

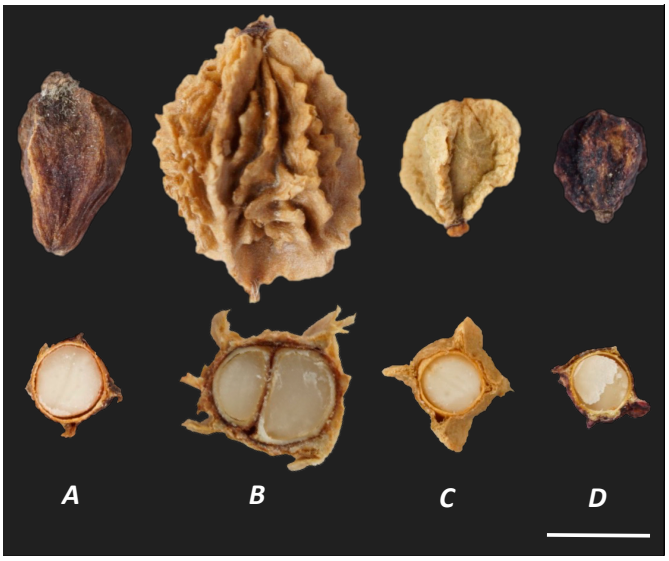

Fig. 2: Seed shapes and cross sections of *S. officinalis* (A), *S. minor* (B), *S. tenuifolia* (C) and *S. parviflora* (D). Scale bare = 2 mm.

oleic acid (C18:1) and < 0.5% eicosenoic acid (C20:1); saturated fatty acids: 4% palmitic acid (C16:0), 2% stearic acid (C18:0), < 2% arachidic acid (C20:0) and behenic acid (C22:0). Thus, linolenic acid, linoleic acid and oleic acid were the predominant fatty acids.

Phenolic constituents

Methanolic crude extracts of the previously defatted seeds of *S. officinalis* were investigated by LC/MS^n. In summary, 18 compounds were tentatively assigned in this fraction based on their retention time (t_R), UV spectra, mass-to-charge ratios (negative ionization mode), as well as their specific fragmentation patterns and corresponding bibliographic references. The chromatogram (Fig. 6) and mass spectra revealed the occurrence of compounds belonging to different substance classes. Among these, hydroxybenzoic acids (methyl-gallate-hexoside), tannins (ellagic acid), flavonoids (quercetin-pentoside, catechin, procyanidins) were assigned, but also *L*-tryptophan, being an aromatic representative of amino acids (Tab. 1). Furthermore, the relative abundance [%] of the assigned compound is shown in Fig. 7.

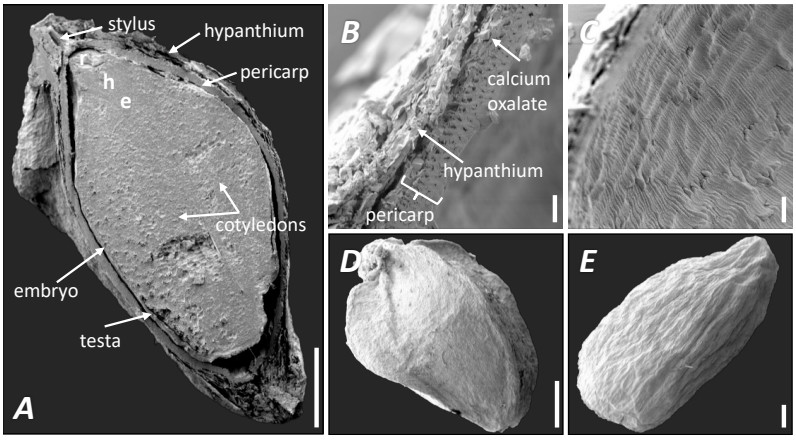

Fig. 3: SEM micrographs of the fruit (seed) of *Sanguisorba officinalis*. A, Longitudinal section. B, Longitudinal section of the perygynous hypanthium and the pericarp with calcium oxalate crystals. C, Inner layer of the pericarp with elongated cells. D, Surface of the fruit. E, Surface of the seed kernel (testa, seed coat). e: epicotyl; h: hypocotyl; r: radicle. Scale bars: A, D = 500 µm, B = 20 µm, C = 50 µm, E = 200 µm.

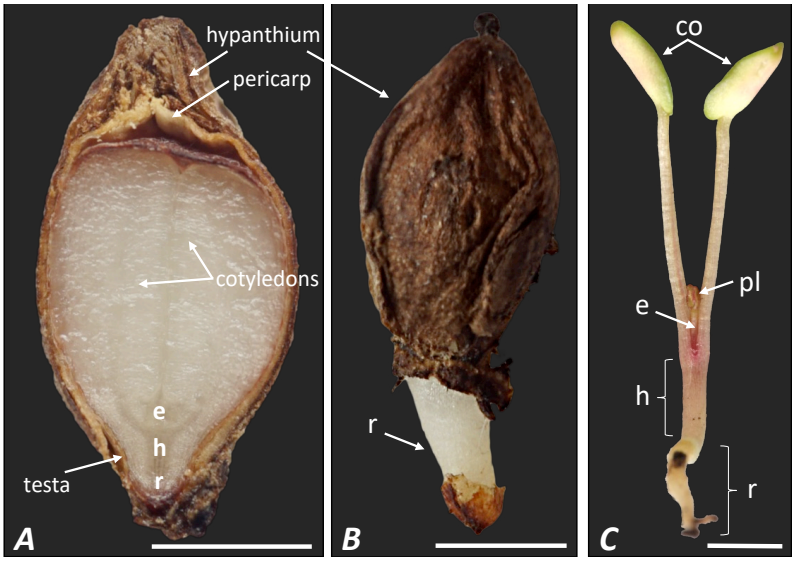

Fig. 4: **A**, longitudinal section of a *S. officinalis* seed after pre-soak in water for 5 days. **B**, germinated seed (*S. officinalis*) with primary root (r) after 8 days. **C**, seedling 14 days after germination. co: cotyledons, e: epicotyl, h: hypocotyl, pl: primary leaves, r: radicle/ root. Scale bars = 1 mm.

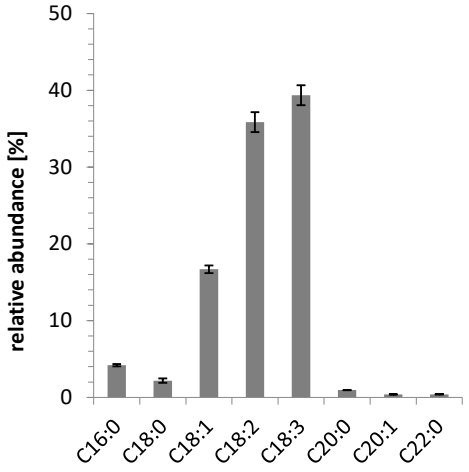

Fig. 5: Fatty acid composition of *S. officinalis* seeds illustrated as mean value of the relative abundance of individual compounds [%] including standard deviation (n=3). Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0).

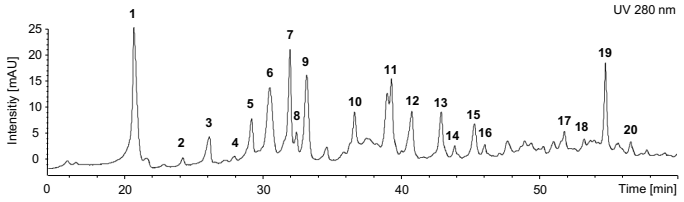

Fig. 6: LC/MS^n chromatogram (UV detection at 280 nm) of phenolic compounds in a MeOH seed extract of *Sanguisorba officinalis*. For compound characterization, cf. Tab. 1.

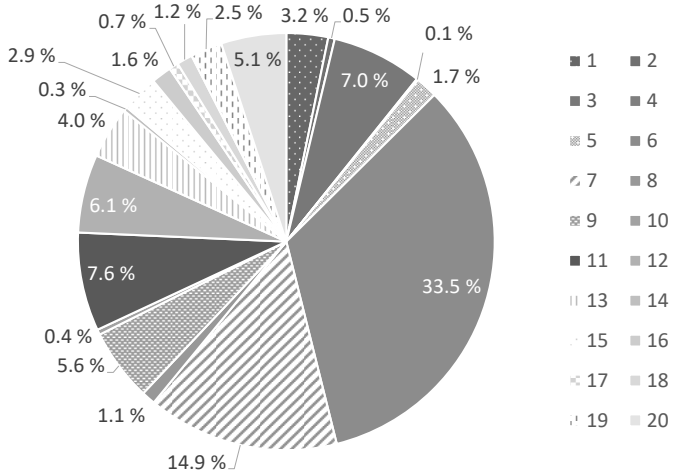

Fig. 7: Phenolic composition of *S. officinalis* seeds illustrated as mean value of the relative abundance of individual compounds [%] (n=2). For compound assignment, see Tab. 1.

Discussion

The dry fruits of *Sanguisorba* form achenes, which persist in dense spikes until late autumn when they shatter, scattering most seeds within a 1 m^2 area around the plant. When harvested, a dry, papery calyx hull surrounds the achenes (Holloway and Matheke, 2003). The fruits of *S. officinalis*, *S. tenuifolia*, and *S. parviflora* (Fig. 1) are ellipsoid to globose, 4-angled, 4-winged with smooth surfaces and one achene. In contrast, the fruits of *S. minor* have serrated netting strips. The serrated surface of *S. minor* appears to be an adaption aiming at the distribution of seeds via the fur of mammals. Large numbers of seeds are distributed by passive attachment to the fur of mammals. As mammals walk through the vegetation, seeds of different sizes, rough surfaces and distributions of hooks are dislodged from the parent plants and attached to the fur (Stiles and Fenner, 2000).

S. officinalis has short, erect botryoids with several single flowers on it. The gynoecium, the female part of a flower, is unicarpellate and includes a stigma, a stylus and a unilocular ovary. It is surrounded by a perigynous hypanthium. Within the locule, a single anatropous unitegmic ovule is formed. After successful fertilization of an inflorescence, the seed (embryo) is formed inside the gynoecium. During that process the inflorescence begins to wither, the bracts and the stamina at the apex of the hypanthium and the stigma dry out and fall off. The fruit (seed, nut) is formed with the dried hypanthium still serving as protection. The gynoecium wall becomes the pericarp during fructification. The inner epidermis of the pericarp is called the endocarp, the outer epidermis is referred to as exocarp, with the mesocarp in between. In the case of nuts, these three layers are equally lignified. The outer layer of *S. officinalis* pericarp, the exocarp, appears to be less lignified, but at the same time containing calcium oxalate crystals (Weddellit, $Ca(C_2O_4) \cdot 2\,H_2O$, (Hartl et al., 2007)). Calcium oxalate widely occurs in plants and may account for 3-80% of plant dry weight (Libert and Franceschi, 1987). As much as 90% of total calcium in a plant may be found as its oxalate salt (Gallaber, 1975). Calcium oxalate is generally considered an end product, and thus its formation reduces the availability of calcium, although some studies have also shown calcium oxalate to be a reversible product (Franceschi and Horner, 1980; Franceschi, 1989; Huang et al., 2015). The occurrence of calcium oxalate crystals in flowering plants is more or less widespread in different plant parts, such as leaves, stems, roots, floral parts, fruits, seeds and pericarps (Mukherjee and Jana, 2014). Calcium oxalate plays an important role in plant defense. Among others, it is an effective deterrent to herbivores.

The embryo is surrounded by a thin seed coat, the testa. The two cotyledons serve as nutritional tissue for germination. To monitor seed germination and development into a seedling, nuts of *S. officinalis* were pre-swollen in water and scattered on soil. After 5 days the cotyledons of the embryo seemed to bulge, and the epicotyl, hypocotyl and radicle started to be developed. After 8 days the radicle of the first seedlings broke off the seed coat, and 14 days after germination small seedlings were developed. Seeds of great burnet (*S. officinalis*) germinate most rapidly at ca. 25 °C after 6 months of dry storage at 4 °C. The cold period is necessary to break seed dormancy. The seeds also germinate without stratification, but then only very sporadically and over a longer period of time (Holloway and Matheke, 2003).

Starch, sugars and fats of the cotyledons provide the energy for germination. The seeds were analyzed by GC/MS to assess their fatty acid profile revealing an average composition typical of members of the Rosaceae family. The study of Matthaus and Özcan (2014) showed that oil contents of 26 varieties of Rosaceae seeds ranged from 3.5 to 46.2 g/100 g. These oils were composed of 3.25-9.17% palmitic, 1.19-4.27% stearic, 6.50-67.11% oleic, 22.08-68.62% linoleic and 0.10-61.59% eicosenoic acids (Matthaus and Özcan, 2014). Based on these wide ranges it is not surprising that the fatty acid profile of *S. officinalis* seeds is within the aforementioned margins (unsaturated fatty acids: 39% linolenic acid (C18:3), 36% linoleic acid (C18:2), 17% oleic acid (C18:1) and < 0.5% eicosenoic acid

Tab. 1: Compound assignment of metabolites detected in MeOH extracts of *S. officinalis* seeds using HPLC-DAD-ESI-MS^n (negative ionization mode).

No.[a]	Constituent	t_R [min]	$\lambda_{max, UV}$ [nm]	$[M-H]^-$ [*m/z*]	Fragmentation ions [*m/z*]	Reference
1	*L*-tryptophan	20.8	218, 280	203	186, 159, 142, 116	[b]
2	1-*O*-caffeoylquinic acid	24.2	324, 374	353	345, 323, 289, 191, 179, 171, 135, 127	(Plazonić et al., 2009)
3	methyl-gallate-hexoside	26.1	214, 270	345	183, 168, 124	(Abu-Reidah et al., 2015)
4	procyanidin B1/B2	27.9	324, 374	578	559, 425, 407, 289, 285, 257	(Bunse et al., 2020)
5	*trans*-3-p-coumaroylquinic acid	29.2	204, 230sh, 312	337	311, 289, 191, 163, 119	[b](Makita et al., 2017)
6	procyanidin	30.3	202, 230sh, 280	577	559, 451, 425, 407, 389, 289, 285, 257, 213	(Bunse et al., 2020)
7	catechin / epicatechin	31.9	204, 230sh, 280	289	245, 227, 203, 187, 175, 161	(Zhao et al., 2013)
8	caffeoylquinic acid	32.4	202, 232, 324	353	289, 250, 191, 173, 127, 85	[b]
9	procyanidin trimer	33.1	202, 230, 280	865	847, 739, 695, 677, 577 543, 525, 391	(Rockenbach et al., 2012)
10	5,6,7-trihydroxy-2,3-dihydrocyclopenta[b] chromene-1,9-dione-3-carboxylic acid	36.5	280, 358, 522	291	247, 219, 203, 191, 175	(Fraternale et al., 2015)
11	*p*-coumaroylquinic acid	39.3	232, 312	337	191, 173, 155, 127, 111, 93, 85	(Bunse et al., 2020)
12	procyanidin dimer	40.7	202, 230, 282	577	451, 425, 407, 389, 289, 285, 257, 213	(Bunse et al., 2020)
13	unidentified gallic acid derivative	42.9	202, 224, 278	497	465, 345, 183, 168, 124	(Hofmann et al., 2016)
14	trigalloyl hexose	43.9	280, 360	635	465, 313, 285, 241, 221, 193, 169	(Abu-Reidah et al., 2015)
15	unidentified gallic acid derivative	45.3	234, 274, 364	497	463, 345, 313, 183, 168, 124	(Hofmann et al., 2016)
16	unidentified	46.0	280, 374	537	519, 339, 195, 179, 161, 143, 119, 89	
17	unidentified	51.7	234, 280, 314	662	644, 602, 516, 456, 438, 324, 307, 248, 205, 163, 145	
18	quercetin-3-O-arabinoside / Dxyloside	53.1	240, 258, 362	433	345, 301, 257, 229, 185	(ReSpect for Phytochemicals, 2020.000Z)
19	ellagic acid	54.7	254, 362	301	284, 257, 245, 229, 201, 185, 165	[b]
20	isoquercetrin	56.5	238, 268, 360	463	301, 271, 255, 229, 193, 179, 151, 107	(Ibrahim et al., 2015)

[a] For peak assignment see Fig. 5. [b] Reference spectra (MoNa, 2020).

(C20:1); saturated fatty acids: 4% palmitic acid (C16:0), 2% stearic acid (C18:0), < 2% arachidic acid (C20:0) and behenic acid (C22:0)).

In addition, the phenolic profile of *S. officinalis* seeds is typical of the rose family (Fahad Al Juhaimi et al., 2016). Hydroxybenzoic acids, tannins and flavonoids belong to the main classes of phenols detected in great burnet herb, roots and flowers (Bunse et al., 2020). Most of these phenolics are bioactive constituents and are exploited for pharmacological purposes as mentioned before. In contrast, the role of these compounds in seeds of *S. officinalis* is still largely unknown and needs further elucidation. However, based on their activity profile, protection of the seeds from bacterial or fungal growth and rot appears conclusive.

Conclusion

This study aimed at a profound characterization of the morphology of *Sanguisorba officinalis* seeds and of their primary and secondary metabolites. For the first time the structure of great burnet seeds was elucidated by scanning electron microscopy. The seed is enclosed by a thin seed coat, which is surrounded by the pericarp and the floral

cup. As a special feature, calcium oxalate crystals were discovered in the outer layer of the lignified pericarp. The occurrence of calcium oxalate crystals in flowering plants is more or less widespread in different plant parts and plays an important role in plant defense. In addition to these morphological studies, the fatty acid and phenolic compound profiles of *S. officinalis* seeds were characterized for the first time. The genus *Sanguisorba* is an important member of the Rosaceae family, the natural habitat of which is becoming increasingly rare. This short study provides a first insight into the seed structure of great burnet and complements previous studies on the flower development of different *Sanguisorba* species (Wang et al., 2020). In the future, further *Sanguisorba* species should be investigated regarding their morphology and phytochemistry to allow direct comparison of taxonomically related species. Furthermore, more detailed information on different compartment structures also considering their specific secondary metabolites may help to deduce the functionality of the latter in the plant. This may also contribute to a better chemotaxonomic differentiation between closely related species or hybrids. In addition, new sources of healthy fatty oils, especially needed for human health and well-being, but also cosmetic applications, are increasingly important. For example, they can supply the body with essential fatty acids or support it in healing processes, e.g. inflammatory skin regions. In the future there could be a greater focus on indigenous medicinal plants cultivated locally, that contain high-quality oils as deduced from their compound profile being abundant in unsaturated fatty acids and revealing the occurence of biologically active phenolics.

Acknowledgement

The authors are grateful to *Prof. Dr. Waltraud X. Schulze* (Department of Plant Systems Biology, Hohenheim University) for the support in creating the manuscript. The authors also gratefully acknowledge *Dr. Rhinaixa Duque-Thüs* (Institute of Botany, Hohenheim University) for identification of the plant specimens and we would like to thank *Dr. Annerose Heller* and her team (Institute of Botany, Hohenheim University) for the great support in teaching microscopy and botany over the last years.

Conflict of interest

The authors reported no potential conflict of interest.

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