The production of 7-methyljuglone, plumbagin and quercetin in wild and cultivated *Drosera rotundifolia* and *Drosera intermedia*

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SUMMARY

The recent establishment of *Sphagnum* farming areas has created large artificial habitats where *Drosera* grows under semi-natural conditions. Here we test the suitability, for pharmaceutical purposes, of two *Drosera* species collected from such areas. We measured the concentration of the biologically active compounds 7-methyljuglone, plumbagin and quercetin in *Drosera rotundifolia* and *D. intermedia*. All three compounds were found in pharmacologically suitable concentrations with 7-methlyjuglone characteristic for *D. rotundifolia* and plumbagin for *D. intermedia*. The concentrations required for pharmacological purposes were achieved within one year, but higher concentrations occurred in older plants and plants in flower. Concentrations did not differ between plants collected in the morning and in the afternoon. *Drosera* plants cultivated under semi-natural conditions are suitable as sources of raw materials for industrial pharmacological applications.

KEY WORDS: collection, cultivation, flavonoids, Droserae herba, naphthoquinones

INTRODUCTION

The European *Drosera* species, round-leaved sundew (*Drosera rotundifolia* L.) and oblong-leaved sundew (*Drosera intermedia* Hayne) are mainly found in nutrient-poor, acid, open wetlands (Juniper *et al*. 1989, Crowder *et al*. 1990, Ellison & Gotelli 2009). The carnivorous plant traps its prey with sticky, sugar-rich mucin droplets exuded from glandular leaf hairs and then digests its prey enzymically (Darwin 1875, Barthlott 2004, Carow 2009). Nitrogen and phosphorous, in particular, are sourced from captured insects. Numerous *Drosera* species are used for medicinal purposes because they produce valuable secondary metabolites, among which the most abundant are two different 1,4-naphthoquinones (Figure 1), namely 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone) and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Kämäräinnen *et al*. 2003). The concentration of these two naphthoquinones differs among *Drosera* species (Krenn & Kartnig 2005). In *D. rotundifolia* 7-methyljuglone is the dominant quinone and plumbagin occurs in only trace amounts. In *D. intermedia* it is the other way round, with plumbagin dominant and 7-methyljuglone occurring in trace amounts only. *Drosera* also contains flavonoids such as quercetin (Šamaj *et al*. 1999) (Figure 1). Many of these secondary metabolites are used in the pharmaceutical, cosmetics and food industries (Banasiuk *et al*. 2012). Several naphthoquinones have been reported to exhibit a wide range of physiological and pharmacological properties. Extracts and tinctures of *Drosera* have anti-inflammatory and spasmolytic effects and are utilised in various medications to treat respiratory diseases (Finnie & van Staden 1993, Blumenthal *et al*. 1998, Krenn *et al*. 2004, Babula *et al*. 2009).

The drug Droserae Herba is traditionally prepared from the dried above-ground parts of *D. rotundifolia* (Egan & van der Kooy 2013). The plants are collected at the beginning of the flowering season, from July to August (HAB 2014, Király *et al*. 2011). Extracts and tinctures from *Drosera rotundifolia* and *D. intermedia* are also used in various medications for the treatment of coughs and pulmonary diseases. In Europe alone some 200–300 registered medications exist that contain *Drosera* as an ingredient (MacKinnon 2009). According to Galambosi (2002) the annual requirement of the European pharmaceutical industry for air-dried *Drosera* biomass is 6–20 tons, of which 1–3 tons is *D. rotundifolia* (Galambosi & Jokela 2002). The increased demand for *D. rotundifolia* and *D. intermedia* in the first part of the 20th century and the destruction of their habitat led to over-exploitation of the wild populations. As the plants have become increasingly rare, they are presently

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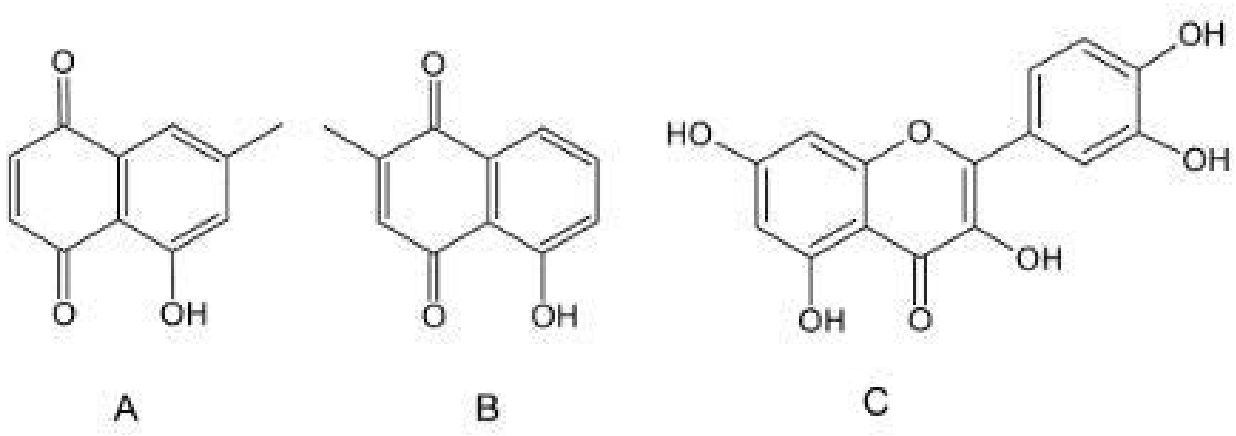

Figure 1. Bioactive compounds from *Drosera* species: (A) 7-methyljuglone, (B) plumbagin, (C) quercetin.

replaced by Asian and African species of *Drosera* (e.g. *D. burmanii* Vahl, *D. peltata* Smith and *D. madagascariensis* DC.) that are officially permitted to be used for pharmaceutical purposes in European countries (Krenn *et al*. 1995, van Wyk *et al*. 2004, Paper *et al*. 2005).

Nowadays, the main commercial source for pharmaceutical sundew material is *D. madagascariensis* DC., which has notably lower concentrations of active ingredients (e.g. 1,4-naphthoquinones, flavonoids and ellagic acid derivatives) compared to *D. rotundifolia* (Krenn *et al*. 1995, Blaschek 1998, Zehl *et al*. 2011, Bäumler 2012).

Current research is increasingly focusing on propagation and cultivation (mainly *in vitro*) of European and non-European sundews. However, these cultivation methods are time-consuming and costly. Recently, the establishment of *Sphagnum* farming areas (Gaudig *et al*. 2014, Krebs *et al*. 2014) has created large artificial habitats where *Drosera* grows in semi-natural conditions. In this article we report work on *Drosera* plants from a *Sphagnum* farming area. Our purposes are:

1. to quantify the biologically active compounds 7-methyljuglone, plumbagin and quercetin;
2. to assess whether the plants have the required minimum concentration of naphthoquinone derivatives; and
3. to determine whether the concentrations of biologically active compounds differ:
   a. over the course of the day;
   b. over the course of the main flowering season (July–August);
   c. with plant age; and
   d. between wild and cultivated populations.

The aim is to identify the most promising populations and harvesting times for sundew.

MATERIALS AND METHODS

Plant material

Entire green parts and roots of *Drosera rotundifolia* and *D. intermedia* were collected in July and August from an artificially established *Sphagnum palustre* L. and *S. papillosum* Lindb. lawn on a rewetted raised bog area near Rastede, NW Germany (53° 15′ 80″ N, 08° 16′ 05″ E). Most of the *Drosera* plants collected (Samples 1–40, 'wild') had germinated from seeds imported, unplanned, with the *Sphagnum* material. A small number of plants (Samples 41–44, 'cultivated') had been established by sowing seeds in cellulose germinating pots placed in the *Sphagnum* lawn. Both 'wild' and 'cultivated' plants were harvested in 2014. A voucher specimen of each species was deposited in the University of Greifswald herbarium under Nos. GFW 51151 (*D. intermedia* Hayne) and GFW 51152 (*D. rotundifolia* L.). Plants of three age groups (≤6 months, 6–12 months, 13–24 months) were harvested at two different times of day (7:00–8:00 h and 15:00–16:00 h) during July and August 2014 (Table 1). Individual plants were grouped by age category using descriptions and images in Nitschke (1860), Drude (1891), Diels (1906) and Bertsch (1912), supplemented by our own observations at the study site.

Extract preparation

The plant material was dried at 40 °C for 72 hours in a Memmert Cleanroom drying oven and ground in a hand-powered drug mill to produce a fine powder. This material (200 mg of each plant) was extracted

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Table 1: Comparison of the 19 test groups. Core criteria are highlighted in bold: **G1** and **G2**: wild *versus* cultivated; **G3–G6**: for both *Drosera* species collection time 07–08 h *versus* 15–16 h; **G7–G12**: for both *Drosera* species plant age ≤ 6 *versus* 6–12 *versus* 13–24 months; **G9**, **G12**, **G13**: for (partially) both *Drosera* species in bloom *versus* not in bloom, **G14–G19**: age of plant at collection. Abbreviations: **x** = naphthoquinone concentration based on either plumbagin or 7-methyljuglone; **7-MJ** = 7-methyljuglone; **P** = plumbagin; **Flav**. = quercetin; **rot** = *Drosera rotundifolia*, **int** = *D. intermedia*.

Group	Samples included	n	Species[1]	Type	Collection time		Age (months)	In flower (%)	Concentration in % dry weight			
					Hour of day	Month			Naphthoquinone	P	7-MJ	Flavonoid
G1	13–16	4	rot	**wild**	07–08	July/August	6–12	0	0.140 ± 0.042		x	0.100 ± 0.055
G2	41 –44	4	rot	**cultivated**	07–08	July/August	6–12	0	0.131 ± 0.022		x	0.097 ± 0,031
G3	2,3,9,10,17,19	6	**rot**	wild	**15–16**	July/August	6–24	0	0.140 ± 0.021		x	0.095 ± 0.027
G4	5,6,13,14,21,23	6	**rot**	wild	**07–08**	July/August	6–24	0	0.147 ± 0.031		x	0.111 ± 0.049
G5	25,27,29,31,33,35	6	**int**	wild	**15–16**	July/August	6–24	0	0.908 ± 0.141	x		0.044 ± 0.013
G6	26,28,30,32,34,36	6	**int**	wild	**07–08**	July/August	6–24	0	0.938 ± 0.125	x		0.044 ± 0.005
G7	2,4,6,7	4	**rot**	wild	15–16 / 07–08	July/August	**≤6**	0	0.129 ± 0.028		x	0.110 ± 0.024
G8	9–12	4	**rot**	wild	15–16	July/August	**6–12**	0	0.143 ± 0.034		x	0.077 ± 0.034
G9	21–24	4	**rot**	wild	07–08	July/August	**13–24**	**30–40**	0.158 ± 0.025		x	0.141 ± 0.038
G10	25–28	4	**int**	wild	15–16 / 07–08	July/August	**≤6**	0	0.752 ± 0.439	x		0.038 ± 0.003
G11	29–32	4	**int**	wild	15–16 / 07–08	July/August	**6–12**	0	0.812 ± 0.155	x		0.048 ± 0.012
G12	33–36	4	**int**	wild	15–16 / 07–08	July/August	**13–24**	**30–40**	0.946 ± 0.091	x		0.047 ± 0.008
G13	37–40	4	**rot**	wild	07–08	July/August	13–24	**60–70**	0.184 ± 0.077		x	0.106 ± 0.045
G14	1,2,5,6	4	rot	wild	15–16 / 07–08	**July**	**≤6**	0	0.109 ± 0.040		x	0.080 ± 0.054
G15	3,4,7,8	4	rot	wild	15–16 / 07–08	**August**	**≤6**	0	0.153 ± 0.026		x	0.087 ± 0.030
G16	9,10,13,14	4	rot	wild	15–16 / 07–08	**July**	**6–12**	0	0.137 ± 0.023		x	0.088 ± 0.033
G17	11,12,15, 16	4	rot	wild	15–16 / 07–08	**August**	**6–12**	0	0.172 ± 0.068		x	0.090 ± 0.059
G18	17,18,21, 22	4	rot	wild	15–16 / 07–08	**July**	**13–24**	0	0.145 ± 0.037		x	0.116 ± 0.030
G19	19,20,23, 24	4	rot	wild	15–16 / 07–08	**August**	**13–24**	0	0.161 ± 0.111		x	0.111 ± 0.057

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three consecutive times (3 hours each) with 10 ml of methanol using a magnetic stirrer (1000 rpm) at room temperature in darkness. The three extracts of each sample were combined, evaporated to dryness, lyophilised and stored at -20 °C.

General analytical procedures

For analytical liquid chromatography-mass spectrometry (LC-MS), a Shimadzu system (pumps LC-20AD, column oven CTO-10ASVP, autosampler SIL-10AF, DAD SPD-M20AD, LCMS-8030 mass spectrometer) using LabSolutions LCMS 5.75 SP2 software was utilised under the following conditions: Synergy 4 µ PolarRP 250 x 4.6 mm (Phenomenex) HPLC column, sample concentration 6 mg ml^{-1} (extract/methanol), injection volume 25 µl, oven temperature 25 °C, gradient elution with acetonitrile/water each acidified with 0.05 % formic acid, gradient sequence (time in min / % acetonitrile) 0/13, 5/16, 26/19.3, 50/80, 51/100, 52/13, 57/13, detection at 254 nm, MS ionisation mode ESI (for quercetin) and Atmospheric Pressure Chemical Ionization (APCI) (for 7-methyljuglone and plumbagin). A single determination was made for each individual sample.

The presence of 7-methyljuglone, plumbagin and quercetin was identified with the help of reference samples and comparison of retention times, UV-spectra and mass spectral data. Quantities were determined by manual integrated peak areas of HPLC-chromatograms using linear equations obtained from calibration with reference samples. Only the main naphthoquinone compounds that could be unambiguously identified by the analysis are included (*D. rotundifolia*: 7-methyljuglone; *D. intermedia*: plumbagin).

Statistical analysis

The results were combined into 19 groups (n = 4 or 6, Table 1) and concentration expressed as mean ± SD. Differences between groups were determined using one-way ANOVA (choosing $P < 0.05$ as statistically significant) and the Kruskal-Wallis test, using R version 3.1.3 (R Development Core Team 2015).

RESULTS

The wild grown (G_1) and cultivated (G_2) *D. rotundifolia* plants showed no statistically significant differences in naphthoquinone (NQ) and flavonoid (Fl) concentration (NQ: $\chi^2 = 0.083$, d.f. = 1, $P = 0.772$, Fl: $\chi^2 = 0$, d.f. = 1, $P = 1$, Kruskal-Wallis test) (Table 2). Highly significant differences were found between the two species (G_3–G_6) in both NQ and Fl concentration ($n = 22$, $P < 0.001$), with *D. intermedia* having six times higher concentrations of NQ than *D. rotundifolia*. No statistically significant differences were found between plants collected at different times during the day (Table 3).

Both species ($G_7 - G_{12}$), showed a consistent increase in NQ and Fl concentrations with age (e.g. *D. rotundifolia* with 0.129, 0.143 and 0.158 % NQ concentration for plants ≤ 6, 6–12, and 13–24 months old, respectively. However the differences between the age classes were not significant ($F = 0.98$; $n = 12$; $P = 0.410$, ANOVA). The NQ concentration also increased with the amount of flowering plants in the sample ($G_{18–19} < G_9 < G_{13}$) but again the differences were not statistically significant ($F = 0.70$; $n = 16$; $P = 0.510$).

In flowering plants the Fl concentration is lower ($G_{18–19} < G_9 > G_{13}$) and the NQ concentration higher ($G_{18–19} < G_9 < G_{13}$) than in non-flowering *Drosera* plants (Table 4). The difference between G_9 (flowering *D. rotundifolia*) and G_{12} (flowering *D. intermedia*) is statistically significant for both NQ and Fl (NQ and Fl: $\chi^2 = 5.333$, d.f. = 1, $P = 0.029$). From July to August, the concentration of bioactive compounds tends to increase (G_{14}–G_{19}, Table 1), but the differences are not statistically significant (NQ and Fl: $n = 24$, $P > 0.05$).

DISCUSSION

This is the first analysis of the concentrations of plumbagin, 7-methyljuglone and quercetin in sundew as a function of time of day and plant age. The time of collection of wild plants determines the concentration and quality of the bioactive compounds and thence the market price of the pharmaceutical drug Droserae Herba. This study shows that the concentration of bioactive compounds in cultivated plants does not differ from those in plants growing spontaneously ('wild'), and are constant over the day so that plants may be collected at any time of day without loss of quality. The concentrations in both *D. rotundifolia* and *D. intermedia* do not differ between July and August, but do increase with age (although not significantly).

The required concentration of naphthoquinones for pharmacological purposes is 0.14–0.22 % (Wichtl 2009). This study shows (Table 1) that, in this semi-natural area, individual *D. rotundifolia* plants may reach this concentration within 12 months, but the majority of plants require 13–24 months. In *D. intermedia* the naphthoquinone (plumbagin) concentration (Table 1) is 1.3–1.6 times the required pharmacological minimum of 0.6 % (Krenn *et al*.

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Table 2: List of individual samples of *Drosera rotundifolia* and *Drosera intermedia*.

No.	Species	Type	Age (months)	Collection time		No.	Species	Type	Age (month)	Collection time	
				Hour of day	Month					Hour of day	Month
1	*D. rotundifolia*	wild	≤6	7:00–8:00	July	23	*D. rotundifolia*	wild	13–24	15:00–16:00	August
2	*D. rotundifolia*	wild	≤6	7:00–8:00	July	24	*D. rotundifolia*	wild	13–24	15:00–16:00	August
3	*D. rotundifolia*	wild	≤6	7:00–8:00	August	25	*D. intermedia*	wild	≤6	7:00–8:00	July
4	*D. rotundifolia*	wild	≤6	7:00–8:00	August	26	*D. intermedia*	wild	≤6	15:00–16:00	July
5	*D. rotundifolia*	wild	≤6	15:00–16:00	July	27	*D. intermedia*	wild	≤6	7:00–8:00	August
6	*D. rotundifolia*	wild	≤6	15:00–16:00	July	28	*D. intermedia*	wild	≤6	15:00–16:00	August
7	*D. rotundifolia*	wild	≤6	15:00–16:00	August	29	*D. intermedia*	wild	6–12	7:00–8:00	July
8	*D. rotundifolia*	wild	≤6	15:00–16:00	August	30	*D. intermedia*	wild	6–12	15:00–16:00	July
9	*D. rotundifolia*	wild	6–12	7:00–8:00	July	31	*D. intermedia*	wild	6–12	7:00–8:00	August
10	*D. rotundifolia*	wild	6–12	7:00–8:00	July	32	*D. intermedia*	wild	6–12	15:00–16:00	August
11	*D. rotundifolia*	wild	6–12	7:00–8:00	August	33	*D. intermedia*	wild	13–24	7:00–8:00	July
12	*D. rotundifolia*	wild	6–12	7:00–8:00	August	34	*D. intermedia*	wild	13–24	15:00–16:00	July
13	*D. rotundifolia*	wild	6–12	15:00–16:00	July	35	*D. intermedia*	wild	13–24	7:00–8:00	August
14	*D. rotundifolia*	wild	6–12	15:00–16:00	July	36	*D. intermedia*	wild	13–24	15:00–16:00	August
15	*D. rotundifolia*	wild	6–12	15:00–16:00	August	37	*D. rotundifolia*	wild	13–24	15:00–16:00	July
16	*D. rotundifolia*	wild	6–12	15:00–16:00	August	38	*D. rotundifolia*	wild	13–24	15:00–16:00	July
17	*D. rotundifolia*	wild	13–24	7:00–8:00	July	39	*D. rotundifolia*	wild	13–24	15:00–16:00	August
18	*D. rotundifolia*	wild	13–24	7:00–8:00	July	40	*D. rotundifolia*	wild	13–24	15:00–16:00	August
19	*D. rotundifolia*	wild	13–24	7:00–8:00	August	41	*D. rotundifolia*	cultivated	6–12	15:00–16:00	July
20	*D. rotundifolia*	wild	13–24	7:00–8:00	August	42	*D. rotundifolia*	cultivated	6–12	15:00–16:00	July
21	*D. rotundifolia*	wild	13–24	15:00–16:00	July	43	*D. rotundifolia*	cultivated	6–12	15:00–16:00	August
22	*D. rotundifolia*	wild	13–24	15:00–16:00	July	44	*D. rotundifolia*	cultivated	6–12	15:00–16:00	August

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Table 3: ANOVA of differences in concentration of naphthoquinones and flavonoid in *Drosera rotundifolia* (G_3–G_4) and *Drosera intermedia* (G_5–G_6) collected at different time of day (G3/G5 = 15–16h, G4/G6 = 07–08h). SS = type III sum of squares; *F* = Fisher's F-value; *P* = probability of significance. Mean values and SE are given in Table 1.

Groups	Naphthoquinone			Flavonoid		
	SS	***F***	***P***	**SS**	***F***	***P***
G3–G4	< 0.001	0.238	0.635	< 0.001	0.534	0.481
G5–G6	< 0.001	0.004	0.948	< 0.001	0.022	0.882

1995) and 135 times more, than the concentration of Droserae Herba sourced from *D. madagascariensis* DC. (Melzig *et al*. 2001). The 7-methyljuglone concentration from samples of flowering *D. rotundifolia* was 6.6–7.6 times higher, than from *D. madagascariensis* DC. (e.g.: 0.024 % in Melzig *et al*. 2001). Maximum concentrations are reached in the samples with 60–70 % flowering plants. Plants younger than one year old can be collected for pharmacological purposes during the flowering season, but efforts are better aimed at collecting older plants, particularly those in flower.

In conclusion, *Drosera* plants cultivated in cellulose germinating pots, placed in the *Sphagnum* lawn, showed no difference in either quality and quantity in regard to the concentration of the biologically active compounds, nor in the time needed for harvest, compared to the *Drosera* plants that occur spontaneously on the Sphagnum farming area. On Sphagnum farming sites cultivated *Drosera* offers new opportunities for the industrial production of *Drosera* under semi-natural conditions.

Table 4: The percentage distribution of naphthoquinone and flavonoid (= quercetin) concentration in 13–24 month-old *D. rotundifolia* plants, with different amount of flowering plants (G18–G19 = 0 %, G9 = 30–40 %, G13 = 60–70 %). Arrowheads show direction of decreasing concentration.

Group	n	Concentration % dry weight	
		Naphthoquinone	**Flavonoid**
G18–G19	8	0.153	0.113
-	-	∧	∧
G9	4	0.158	0.141
-	-	∧	∨
G13	4	0.184	0.106

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