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Main constituents of a commercial *Drosera* fluid extract and their antagonist activity at muscarinic M_3 receptors in guinea-pig ileum

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The range of known constituents of *Drosera* species is extended by identification of myricetin 3-*O*-galactoside, from *D. madagascariensis*, and (+)-*cis*-isoshinanolone, obtained from a commercial fluid extract. They are accompanied by the naphthoquinones droserone and plumbagin, typical of this taxon, and a series of ubiquitous flavonols, including the rarely found gossypitrin present in the latter source. Conspicuously, the commercial form of *D. peltata*, non-accepted by the commission E, was found to be devoid of flavonoids. In addition, the fortuitous availability of the authentic enigmatic sample 'CON', previously isolated from *D. rotundifolia*, led to its characterization as common quercetin. Experiments performed on isolated guinea-pig ileum demonstrated that quercetin respectively 'CON' moderately inhibited carbachol-induced contractions at 10 μM (pD'$_2$ 5.09 ± 0.02), while (+)-*cis*-isoshinanolone (100 μM) was inactive. This result indicates that quercetin derivatives may well contribute to the therapeutic use of *Drosera* preparations.

1. Introduction

The use of *Drosera* species including *D. rotundifolia*, *D. ramentacea*, *D. intermedia*, *D. peltata* and *D. madagascariensis* as a general remedy for the treatment of whooping cough in traditional medicine, initiated numerous chemical and pharmacological investigations of the constituents of the medicinally used species [1, 2]. Previous work on *Drosera* species revealed the presence of a variety of naphthoquinones and indicated the presence of flavonoids. From pharmacological studies and traditional medical practices, the naphthoquinones are of particular interest, representing the alleged biologically active substances. In an earlier communication on the constituents and biological activity of *D. rotundifolia*, an enigmatic isolate possessing pronounced antitussive and spasmolytic activity was reported [3]. It was claimed to be a 'carboxy-oxy-naphthoquinone' and thus designated as 'CON'. Since then, the efficacy of *Drosera* preparations was, at least in part, attributed to the presence of this mysterious naphthoquinone metabolite. The fortuitous delivery of the authentic sample of 'CON' prompted the present investigation of metabolites of a commercial *Drosera* fluid extract, including the structural and pharmacological re-examination of 'CON'.

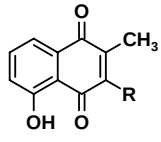
2. Investigations, results and discussion

2.1. Phytochemical studies

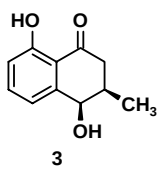
Initial separation of components in the *Drosera* fluid extract was achieved by successive partitions between water and organic solvents. Subsequent combined fractionation procedures of the individual extractives of the petrol ether, dichloromethane and ethyl acetate phases based on the use of Sephadex LH-20, silica gel and polyamide as chromatographic substrates with various solvent systems succeeded in affording compounds (**1**–**9**), including a tetralone derivative not previously encountered in the genus *Drosera*.

Structural examination of the petrol ether extractives revealed three naphthoquinones, visualized by the typical colour on TLC plates and the response to the anisaldehyde-H_2SO_4 reagent. Amongst these, droserone (**1**) and plumbagin (**2**) represent known metabolites of the above class of secondary products that are typical of the plant family Droseraceae. These compounds were readily identified by means of their spectroscopic data (MS, ^{1}H and ^{13}C NMR) which were identical to those of authentic specimens; the latter is reputed for its broad spectrum of biological and pharmacological properties [4].

Naphthoquinone **3,** exhibiting chromatographic mobilitiy similar to that of **1**, was isolated as a yellow oil and gave a greyish colouration with the anisaldehyde-H_2SO_4 reagent. Its EI-MS showed a $[M]^+$ at *m/z* 192, consistent with a molecular formula $C_{11}H_{12}O_3$ for the metabolite. Treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) converted **3** into plumbagin (**2**), identified by comparison with a reference sample. Analysis of the ^{1}H NMR spectrum of **3** revealed the presence of an AMX spin system (δ 7.52, *dd*, J 7.4 and 7.6 Hz, H-6; δ7.00, *d*, J 7.4 Hz, H-7; δ 6.83, *d*, J 7.6 Hz, H-8) and a sharp low-field singlet at δ 12.43 (5-OH), reminiscent of the aromatic portion of the structure of **1**. Besides a methylene group, the aliphatic region exhibited two methine signals at δ 2.44 (H-3) and 4.77 (H-4), the latter coupled to an oxygen-bearing aliphatic carbon (δ_c 70.9). Location of a methyl group at C-3 and full assignment of signals were achieved by ^{1}H-^{1}H COSY, HMQC and HMBC experiments. The relative 3,4-*cis* configuration of **3** was evident from the ^{1}H NMR coupling constants of the corresponding protons ($^3J_{3,4}$ 2.9 Hz). These spectral features identified **3** as *cis*-isoshinanolone, an acetogenic metabolite of wide-spread occurrence in higher plants [5]. The remaining problem concerned the absolute configuration at these



1 R = OH
2 R = H



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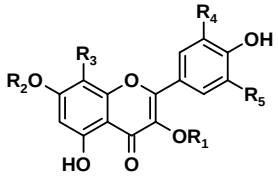

	R_1	R_2	R_3	R_4	R_5
4	H	H	H	H	H
5	H	H	H	OH	H
6	Galactosyl	H	H	OH	H
7	Glucosyl	H	H	OH	H
8	H	H	OH	OH	H
9	H	Glucosyl	OH	OH	H
10	Galactosyl	H	H	OH	OH

chiral centres which cannot unambiguously be deduced from the optical rotation due to the large variability of reported [α]-values. Although the optical rotations (see Experimental) strongly indicated the presence of the (3*R*, 4*R*)-enantiomer, confirmation of this conjecture was provided by circular dichroism (CD). Following recent establishment of the absolute structure of *cis*- and *trans*-isoshinanolones by an HPLC-CD analysis [6], the strong negative Cotton effect at 214 nm and the positive one at 258 nm in its CD-spectrum provided evidence for the (3*R*, 4*R*)-configuration. Compound **3** was, therefore, identified as (+)-*cis*-isoshinanolone, its presence in a fluid extract of the processed aerial parts of this natural source being now demonstrated for the first time.

The ethyl acetate and dichloromethane extract afforded a series of flavonols (**4**–**9**). Comparison of the ^{1}H NMR spectra of the common flavonols **4**–**7** with those of reference compounds commercially available, established their identity. In addition, the extracts also afforded gossypetin (**8**) and its rarely found 7-*O* glucoside (gossypitrin) (**9**) [7], their structural assessment being effected by means of spectroscopic methods (MS, ^{1}H NMR) and acid hydrolysis. These metabolites suggested that the origin of the examined fluid extract is strongly associated with *D. rotundifolia*, as it was claimed.

The fortuitous delivery of the authentic sample of 'CON', which is of special interest as part of the underlying active principle of *Drosera* preparations since many years (*vide supra*), facilitated definition of its chemical nature. Following extensive spectroscopic studies (^{1}H and ^{13}C NMR, MS, UV), the structure of the mysterious isolate 'CON' was unambiguously established to be that of common quercetin (**5**). Initially, this very unexpected result called into question the authenticity of the delivered specimen. Based on the explicit re-confirmation that the sample in question is an original and personal gift from Krahl, we feel confident of its genuineness. Further, the failure of both the re-isolation of the a priori structurally unknown 'CON' from the same plant source by other authors and the identification of a carboxy-oxy-naphthoquinone-type compound from any *Drosera* species should also give credence to the present outcome.

In the continuation of this work we have thus extended our study to a number of commercial *Drosera* drugs with emphasis on the flavonoid profiles, including *D. longifolia, D. intermediate, D. ramentacea, D. madagascariensis* and *D. peltata* as claimed by the declaration. According to expert advice [8], the taxonomic identity was only confirmed for *D. peltata*, while the remaining plant material with an identical flavonoid pattern represented *D. madagascariensis*, irrespective of the declaration. This finding emphasizes once more the necessity of the unambiguous taxonomic identification of plant materials. Concerning *D. madagascariensis*, the flavonoid profile comprised compounds **5**–**7** and myricetin 3-*O*-galactoside (**10**), which was not previously encountered in the genus *Drosera*. Its structure was similarly confirmed by a combination of hydrolytic studies and spectroscopic methods. In contrast to previous reports [9], flavonoids appeared to be absent in the methanol extract of *D. peltata*. This finding provides a chemical basis for the non-acceptance of this species by the commission E.

2.2. Pharmacological data

Given the structure elucidation of 'CON' (**5**; *vide supra*) and having in mind its potential role in broncholytic and related spasmolytic conditions [1, 2], we next examined the spasmolytic activity of **5** and its associated glycoside isoquercitrin (**7**) using a guinea-pig ileum model. The documented activity of naphthoquinones prompted us to include the new tetralone derivative **3** and the naphthoquinone **2** in this pharmacological study. These compounds were investigated as antagonists of carbachol-induced contractions in guinea-pig ileum (Fig.). The maximal contractile response to carbachol was depressed to 45 ± 1% in the presence of quercetin (**5**) (10 μM). On the other hand, **5** failed to shift the concentration-effect curve to carbachol to the right. Thus, **5** proved to be a weak non-competitive antagonist of carbachol-induced contractions [$pD'_2 = 5.09 \pm 0.02$ ($n = 4$)]. This observation is consistent with previously reported findings in guinea-pig ileum [10]. Compound **3** (1–100 μM) failed to produce a depression of the concentration-effect curve to carbachol and showed minimal antagonist activity [$pA_2 = 3.90 \pm 0.10$ ($n = 4$)]. Isoquercitrin (**7**) (100 μM) shifted the concentration-effect curve to carbachol to the right and evoked a depression of the maximal response to 85 ± 3% [$pA_2 = 4.58 \pm 0.06$ ($n = 6$)]. In contrast, plumbagin (**2**) (10 μM) failed to shift the concentration-effect curve to carbachol to the right and behaved as a weak non-competitive antagonist of carbachol-induced contractions [pD'_2 was 4.44 ± 0.15 ($n = 4$)].

From these findings, it can be concluded that quercetin (**5**) and its glycosides play an important role in the therapeutic

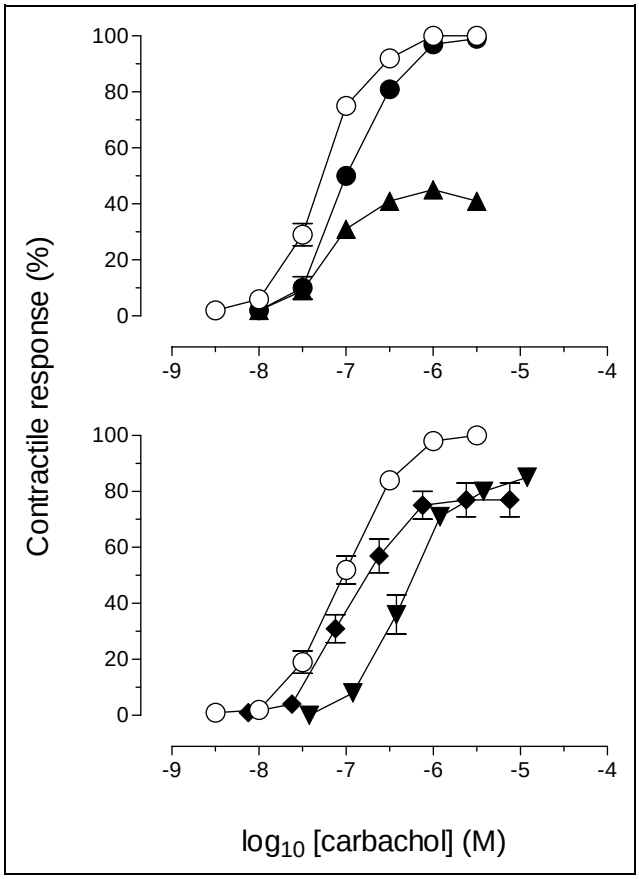

Fig.: Concentration effect-curves to carbachol in guinea-pig-ileum (whole segments) in the absence (○, $n = 8$) and presence of 100 μM **3** (●, $n = 4$) and 10 μM **5** (▲, $n = 4$) (*upper panel*), and in the absence (○, $n = 10$) and presence of 100 μM **7** (▼, $n = 6$) and 10 μM **2** (◆, $n = 4$) (*lower panel*). Effects are expressed as a percentage of the maximal contractile response to carbachol in the first curve. Values are mean ± SEM (vertical bars, only illustrated when larger than symbols).

application of *Drosera*-containing preparations, provided that there are no bioavailability problems. Against this background, the relatively high content of quercetin derivatives, being in the range of ca. 2% of individual representatives [11], deserves to be explicitly mentioned. In contrast, the absence of similar spasmolytic activity of plumbagin (**2**) and the tetralone derivative (**3**) suggests that naphthoquinones and related compounds are less significant as far as the underlying active principle of *Drosera* is concerned, taking also into account the low naphthoquinone concentration in this plant source [12].

3. Experimental

3.1. Materials

The *Drosera* fluid extract was obtained from Gehrlicher, Pharmazeutische Extrakte, Eurasburg, Germany. Commercial samples of *Drosera* species were from Finzelberg, Andernach (*D. madagascariensis, D. peltata*), Gehrlicher, Pharmazeutische Extrakte, Eurasburg, Germany (*D. longifolia*) and Müggenburg GmbH & Co, Alveslohe, Germany (*D. ramentacea, D. peltata*). Voucher specimens have been deposited at the Institut für Pharmazie, Pharmazeutische Biologie, Freie Universität Berlin. Authentic 'CON' was supplied by M. Elzer (Fa. Makara, Aldenhoven). Carbachol was purchased from Sigma, München, Germany. All other reagents were obtained from various commercial suppliers.

3.2. Extraction and isolation

3.2.1. Fluid extract

The *Drosera* fluid extract (850 ml) was reduced in volume to remove EtOH, and the residual aqueous phase successively extracted with petrol ether (5 l), CH_2Cl_2 (3 l), and EtOAc (2 l). The petrol ether extractives (1 g) were initially chromatographed on silica (80×2 cm; petrol ether/EtOAc 19 : 1; flow rate 0.5 ml/min) and the subfractions 165–260 further purified on Sephadex LH-20 with MeOH as eluant. The content of test tubes 10–17 contained a mixture, which was resolved by prep. TLC (petrol ether/EtOAc 7 : 3) to give two bands at R_f 0.52 and 0.82, respectively.

Compound **1**: R_f 0.52 (26 mg), EI-MS $[M]^+$ 204 (100%); ^{1}H NMR (300 MHz, CD_3OD): δ 1.93 (*s*, 3-C<u>H</u>$_3$), 7.00 (*dd,* J 8.1 and 1.4 Hz, H-7), 7.45 (*dd*, J 7.4 and 1.4 Hz, H-5), 7.51 (*dd*, J 7.5 and 8.1 Hz, H-6). ^{13}C NMR (75 MHz, CD_3OD): δ 9.0 (3-<u>C</u>H$_3$), 115.7, 118.3, 118.6, 121.8, 136,7, 137.4, 162.3, 170.2, 184.6, 191.4.

Compound **2**: R_f 0.82 (27 mg), EI-MS $[M]^+$ 188; ^{1}H NMR (300 MHz, acetone-d_6) δ: 2.17 (*d*, J = 1.5, 2-CH_3), 6.88 (1 H, d, J = 1.5, H-3), 7.3–7.7 (3 H, m, H-6–H-8), 12.00 (1 H, s, 5-OH).

Compound **3**: The content of tubes 350–430 afforded the tetralone derivative **3** (130 mg); R_f 0.54; EI-MS $[M]^+$ 192 (99); $[\alpha]_D$ + 63.7° (c = 0.72, acetone), $[\alpha]_D$ + 144° (c = 1.0, MeOH); UV (MeOH) λ_{max} nm (log ε) 231 (1.89), 306 (0.84). ^{1}H-NMR (300MHz, acetone-d_6) δ: 1.11 (3 H, *d*, J 6.8 Hz, 3-C<u>H</u>$_3$), 2.44 (1 H, *m*, H-3), 2.55 (1 H, *dd*, J 17.5 and 4.3 Hz, H-2_{eq}), 2.85 (1 H, *dd*, J 17.5 and 10.1 Hz, H-2_{ax}), 4.77 (1 H, *d*, J 2.9 Hz, H-4), 6.83 (1 H, *d*, J 7.5 Hz, H-8), 7.00 (1 H, *d*, J 7.4 Hz, H-7), 7.52 (1 H, *dd*, J 7.6 Hz, H-6), 12.43 (1 H, *s*, 5-OH). ^{13}C NMR (75 MHz, acetone-d_6) δ: 16.2 (C-11), 35.6 (C-3), 41.7 (C-2), 70.9 (C-4), 147.6 (C-9), 117.5 (C-7), 119.7 (C-5), 115.8 (C-10), 137.5 (C-6), 163.2 (C-8), 206.3 (C-1). CD (MeOH): $[\Phi]_{214}$ + 15000, $[\Phi]_{230}$ + 1000, $[\Phi]_{236}$ − 350 $[\Phi]_{258}$ + 4100.

The CH_2Cl_2 soluble portion (7.6 g) was chromatographed on Sephadex LH-20 with MeOH as eluant to afford the known flavonols **4**–**8**.

Compound **9**: The EtOAc soluble extractives (11 g) were similarly separated on Sephadex LH-20 using a linear gradient system of H_2O/MeOH (1 : 4 → 0 : 1) to yield compound **9** (3 mg). MS: $[M]^+$ 480. ^{1}H NMR (360 MHz, DMSO-d_6) δ: 6.63 (s, H-6), 6.91 (d, J = 7.9 Hz, H-5′), 7.64 (dd, J = 9.0 and 2.5 Hz, H-6′), 7.77 (d, J = 2.5 Hz, H-2′). ^{13}C NMR (DMSO-d_6) δ: 60.6, 69.6, 73.1, 75.6, 77.2, 97.8, 101.4, 104.6, 115.2, 115.5, 120.2, 122.0, 126.8, 135.7, 143.5, 145.0, 147.3, 147.8,150.2, 151.4, 176.2.

3.2.2. Commercial drugs

The commercial forms of each *Drosera* species (100 g) was successively extracted with CH_2Cl_2 and MeOH (1.4 l in each instance) using a Soxhlet apparatus. The methanol extractives were subjected to chromatography on Sephadex LH-20 (80×2.5 cm; MeOH, 2000 ml, MeOH/Me_2CO 1 : 1, 400 ml; flow rate 0.6 ml/min); fractions (10 ml) were collected and combined according to their TLC behaviour. Tubes 45–52 contained **5**–**7** (34 mg), which were resolved by HPLC (MN nucleosil 100 C 18; 25×0.4 cm; flow rate 0.9 ml/min) using a linear gradient system of 5% HOAc-MeOH containing increasing amounts of MeOH.

Compound **10:** The content of fractions 56–60 was rechromatographed on Sephadel LH-20 eluting with a linear gradient system of H_2O/MeOH as noted above; tubes 73–78 (9 mg). UV (MeOH) λ_{max} nm 259, 365; (+ NaOMe) 397; (+ NaOAc) 273, 385; (+ NaOAc/H_3BO_3) 260, 387; (+ $AlCl_3$) 265, 397; (+ $AlCl_3$/HCl) 270, 407. MS: $[M]^+$ 480. ^{1}H-NMR (300MHz, DMSO-d_6) δ: 3.3–3.6 (m, H-2″–H-6″), 5.34 (d, J = 7.7 Hz, H-1″), 6.19 (d, J = 1.7 Hz, H-6), 6.37 (d, J = 1.7 Hz, H-8),7.21 (s, H-2′ and H-6′), 12.64 (br s, 5-OH).

3.3. Antagonist activity of samples at muscarinic M_3 receptors in guinea-pig ileum

Guinea-pigs of either sex, 300–450 g, were stunned by a blow on the neck and bled. Whole segments of the ileum (1.5 cm in length) were mounted isotonically in 20-ml organ baths filled with Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 11.0 and glucose 5.6. The solution was gassed with 95% O_2–5% CO_2 and warmed to a constant temperature of 37 °C (pH, 7.4). After a stabilization period of 20 min the segments were stimulated three – four times with carbachol (1 μM) during a period of 45 min to establish a constant response. Up to four cumulative concentration-effect curves to carbachol were constructed at intervals of 15–20 min in the absence and presence of antagonist. Antagonists were incubated for 5–10 min. It has previously been shown that successive concentration-effect curves to carbachol (control curves) are highly reproducible in the guinea-pig ileum assay [13]. For atropine, a pK_B value of 9.02 ± 0.06 (slope 1.05 ± 0.03 of Schild plot, $n = 30$) was obtained [13].

Results are given as means ± SEM. Antagonist affinities were expressed as apparent pA_2 values, which were calculated from the equation:

$$pA_2 = pA_X + \log (r - 1) \quad (1)$$

where pA_X is the negative logarithm of the concentration of antagonist used and r (concentration ratio) is the ratio of agonist EC_{50} measured in the presence and absence of antagonist [14]. Antagonists which induced a depression of the maximum response to the agonist without shifting the concentration-effect curve to the right (non-competitive antagonists) were characterized by estimating the pD_2' value according to the equation:

$$pD_2' = pA_X + \log (100/E_{max} - 1) \quad (2)$$

where pA_X is the negative logarithm of the concentration of antagonist used, and E_{max} the maximum response observed in the presence of antagonist as percentage of the maximum response elicited by the agonist in the absence of antagonist [15].

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