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Lipid and Phenolic Constituents from Seeds of *Hypericum perforatum* L. and *Hypericum tetrapterum* Fr. and their Antioxidant Activity

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Seeds of *Hypericum perforatum* and *H. tetrapterum* were extracted with dichloromethane and methanol and investigated by chromatographic and mass spectrometric methods. Both species yielded a fatty oil fraction amounting to 30.5 % and 18.0 % of the seed weight, respectively. Linoleic acid (C18:2n-6) was shown to be the predominant fatty acid constituent. Moreover, xanthone derivatives, *i.e.* tetrahydroxyxanthones (THX), xanthone-glycosides and xanthone-sulfonates, were assigned in methanolic extracts. For structure elucidation, one representative xanthone, namely 1,3,6,7-THX, was synthesized and analyzed *via* HPLC-DAD-MS^n and GC-MS. Total THX contents were quantitated applying a validated HPLC-DAD method, resulting in 1.25 g/kg (*H. perforatum*) and 0.27 g/kg (*H. tetrapterum*), respectively. Moreover, the free radical scavenging capacity of the methanol extracts was tested using the DPPH antioxidant assay. Both, *H. perforatum* (IC_{50} = 8.7 mg/L) and 1,3,6,7-THX (IC_{50} = 3.0 mg/L), exhibited good DPPH free radical scavenging activity compared to *Trolox* (IC_{50} = 6.6 mg/L).

Keywords: Antioxidants • Fatty acids • St. John's wort • Seeds• Xanthone derivatives

Introduction

The genus *Hypericum* includes more than 400 species, which are distributed all over the world [1; 2]. The most intensively studied representative of this genus, *Hypericum perforatum* L. (St. John's wort, SJW), is widely used in phytomedicine *e.g.* for the symptomatic treatment of mild and moderate depressive episodes [3-6]. Besides its antidepressant activity, several additional pharmacological effects have been documented for *H. perforatum*, including among others antiviral, antibacterial and woundhealing effects [7-10], which have continuingly stimulated investigations into *H. perforatum* in particular and other *Hypericum species* in the past [1]. Belonging to the same genus, the perennial plant *Hypericum tetrapterum* Fr., also known as St.

Peter's wort, has been investigated less thoroughly. However, a broad spectrum of biofunctionalities, including antimicrobial activity and antioxidant capacity, has been determined for crude methanol extracts prepared thereof [11; 12].

The aerial parts of *Hypericum* plants contain a wide spectrum of secondary metabolites, including acylated phloroglucinol derivatives (*e.g.* hyperforin and adhyperforin), naphthodianthrones (*e.g.* hypericin and pseudohypericin), flavan-3-ols (*e.g.* catechin) and further flavonoids (*e.g.* hyperoside, quercetin, amentoflavone and I3,II8 biapigenin) [13-15]. In contrast, subterraneous parts of *Hypericum* received less attention, even though they are known to contain phenolic acids, flavonols, flavan-3-ols and xanthones [16-18]. As interesting natural plant constituents, xanthones hold a distinct position in the phytochemistry and pharmacology of *Hypericum* [19]. Specifically, several structural types, *e.g.* oxygenated, prenylated and glycosylated xanthones, which were primarily found in roots of the plant, have been isolated from various *Hypericum* species [16; 20].

Only recently, novel polyprenylated tetraoxygenated xanthones have been detected in roots of *H. monogynum*. Additionally, xanthone derivatives have also been extracted from suspended tissue cultures and calli [21-23]. Furthermore, Dias *et al*. (2000) [24] investigated the biosynthesis of oxygenated xanthones in *H. androsaemum* and obtained high levels of 1,3,5,6- and 1,3,6,7-THX. Ferrari *et al*. (2006) [22] firstly isolated 1-hydroxy-5,6,7-trimethoxyxanthone and 3-*O*-methylpaxanthone from calli of *H. perforatum*. Various biological properties have been attributed to xanthones in previous studies [25]. Antimicrobial, antioxidant [23] as well as anti-inflammatory activities [16] have been described, besides other pharmacological effects such as an anticarcinogenic [26], antitumor and neuroprotective potential [27].

Since data for seeds of both *H. perforatum* and *H. tetrapterum* are scant in literature [28; 29], the aim of the present study was to systematically investigate the phytochemical profile of representative samples of seeds from both species, providing a solid basis for pursuing future routine control testing. Plant seeds are generally known to contain a wide range of substances with antioxidant activities, *e.g.* phenolic compounds such as hydroxylated derivatives of benzoic acids, coumarins, flavonoids or lignans [30]. Consequently, seeds of *Hypericum* were comprehensively analyzed to complete the knowledge of phytochemical constituents of *Hypericum*. In addition, the present study should lay the foundation for evaluating, whether a chemotaxonomic differentiation of *Hypericum* species is possible, when focusing on the seed composition. For this purpose, we systematically compared the seeds of the aforementioned *Hypericum* species with regard to their lipophilic compounds, their phenolic secondary metabolites as well as their antioxidant activity.

Results and Discussion

For the comprehensive investigation of *H. perforatum* and *H. tetrapterum* seeds, a two-step extraction with solvents of different polarities und extraction capacities, *i.e.* CH_2Cl_2 and MeOH, was performed. The extracts thus obtained were further analyzed *via* HPLC-DAD-MS^n and GC-MS. In this course, a fragmentation mechanism of 1,3,6,7-THX, both in ESI negative ionization mode and also in EI mode, when analyzed as TMSi derivative, was proposed. For the comparison of extraction yields, THX were quantitated. Moreover, the potential of methanolic seed extracts of *H. perforatum* and *H. tetrapterum* to scavenge free radicals was tested in the DPPH antioxidant assay.

Lipophilic constituents of H. perforatum and H. tetrapterum seeds

Overall, seeds of *H. perforatum* and *H. tetrapterum* contain a substantial portion of a bright yellow fatty oil. Recovery of the latter was performed applying a two-step extraction with dichloromethane resulting in yields of 30.5 % and 18.0 %, respectively. The dichloromethane extracts were further analyzed after derivatization *via* GC-MS in order to obtain a comparative overview of the lipophilic seed components. A series of saturated and unsaturated fatty acids were detected as methyl esters, ranging from palmitic acid (C16:0) to linolenic acid (C18:3), whereas linoleic acid (C18:2) was found to be the predominant fatty acid of both species. As deduced from relative peak areas, relative proportions of linoleic acid and linolenic acid for *H. perforatum* were calculated as 43.2 % and 36.2 % and for *H. tetrapterum* as 44.1 % and 31.3 %, respectively. Relative peak areas of the detected

fatty acids are summarized in *Figure 1*. Additionally, β-sitosterol, behenic acid (C22:0) as well as unidentified monoglycerides were identified as minor components in seed oils of both investigated species.

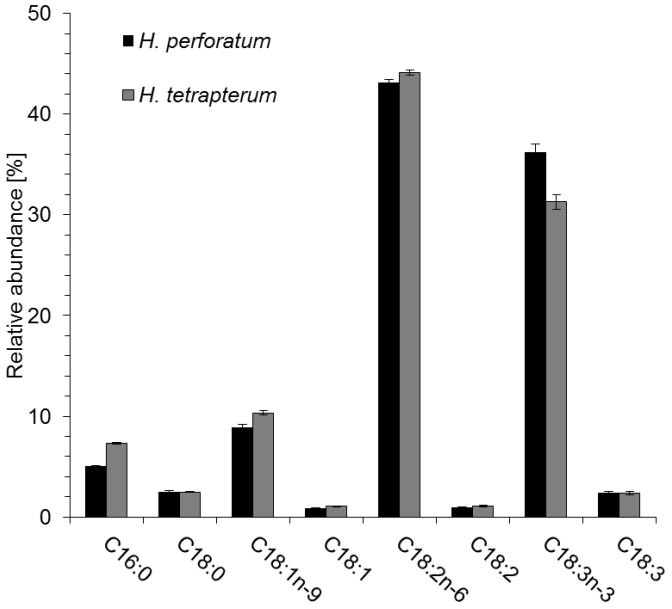


Figure 1. Fatty acid profiles of *H. perforatum* and *H. tetrapterum* seeds illustrated as relative peak areas. Bars represent means ± standard deviation (*n*=3).

Chromatographic analyses of methanolic seed extracts

For the determination of polar constituents *Hypericum* seeds were further extracted with methanol and subsequently analyzed *via* HPLC-DAD-MS^n. *Figure 2* illustrates the characteristic UV-chromatographic profile of both species, recorded at 260 nm. Moreover, the methanolic extracts were further evaluated *via* GC-MS, obtaining a comprehensive view of polar plant secondary metabolites.

As can be deduced from *Figure 3*, a series of sugars and xanthones were identified. By comparing the mass spectra of the respective signals with those of reference standards and with the mass spectra of the NIST database, the disaccharide sucrose and the trisaccharide raffinose were identified. Hence, **S1** was assigned to the sucrose-TMSi derivative and **S5** to the raffinose-TMSi derivative, respectively. The tetrahydroxyxanthones (**X1**-**X3**) detected by GC-MS were corroborated by LC-MS analyses (*Figure 2, Figure 3*). For structure elucidation and comparison, 1,3,6,7-THX and 1,3,5,6-THX were synthesized according to literature protocols, and, additionally, their structure was confirmed by their ^{1}H- and ^{13}C-NMR signals. The determination of the THX configuration is demanding, since their UV data and also mass spectroscopic behavior are highly similar, due to their identical molecular weight. Surprisingly, 1,3,5,6-THX was detected neither in the seeds of *H. perforatum,* nor in those of *H. tetrapterum*. However, the synthesized 1,3,6,7-THX was congruent with **X3** in its chromatographic, UV and mass spectrometric behavior. Our findings are in contrast to previously published data, concerning the configuration of the two main THX so far analyzed in *Hypericum.* One explanation for this differing xanthone profile might be the fact, that herbal and root materials were analyzed in these previous works, whereas seeds were studied in the present investigations. Additionally, the configuration of the THX was non-exhaustively proven by NMR experiments or reference standards in the past.

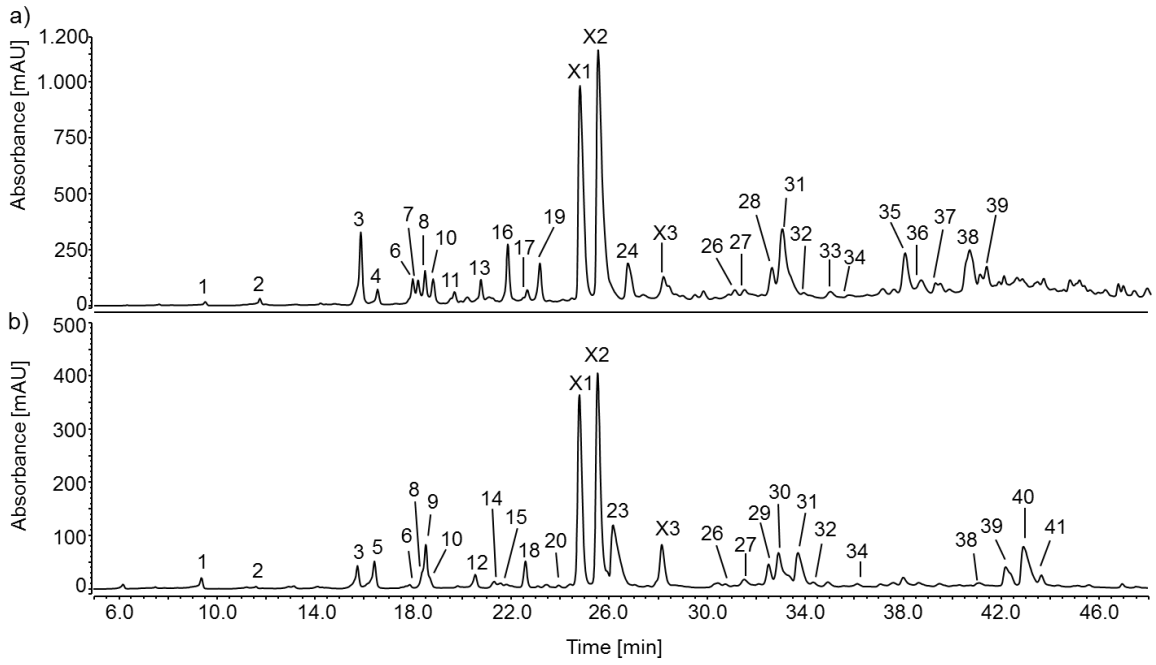


Figure 2. Chromatographic profiles (HPLC-DAD) of methanolic seed extracts of a) *H. perforatum* and b) *H. tetrapterum* recorded at 260 nm. For peak assignment cf.

Table 1

Moreover, retention indices were calculated for **X1**, **X2** and **X3**, allowing a valuable comparison of signals under varying laboratory conditions [31]. In brief, by using the respective homologous *n*-alkanes, eluting directly prior to and after the target component, retention indices were determined as follows: 2813.0 (**X1**), 2887.3 (**X2**) and 2929.9 (**X3**). Expectedly, differences in the fragmentation patterns of **X3** in the EI-MS and ESI^--MS modes were observed (*Figure 4*). A fragmentation mechanism of 1,3,6,7-THX, both in ESI negative ionization mode and also in EI mode, when analyzed as TMSi-derivative, was proposed (*Scheme 1*). As shown in *Figure 4a)*, the molecular ion peak of the TMSi-derivative (*m/z* 548) was of very low intensity. Release of a methyl group (loss of 15 Da) yielded a base peak fragment at *m/z* 533. Subsequently, the loss of a trimethylsilyl moiety (73 Da) followed by another loss of a methyl group (15 Da) resulted in a minor fragment at *m/z* 445. Additionally, fragments at *m/z* 415 and 373 were detected as a result of a dimethyl (30 Da) and a subsequent methylidynesilane (42 Da) cleavage, respectively. In comparison, in ESI^--ionization mode, the molecular ion peak at *m/z* 259 was observed without any further derivatization, which is in agreement with literature data [19]. This ion species revealed the successive losses of two CO molecules (28 Da). The resulting observed mass spectrometric species at *m/z* 203 is supposed, without any further spectral evidence, to be a substituted cycloheptatriene anion. Concurrently, being in accordance with a fragmentation pathway described in literature for monomeric xanthone aglycones [32], the loss of a CO_2 molecule (44 Da) followed by the release of a CO molecule (28 Da) occurred.

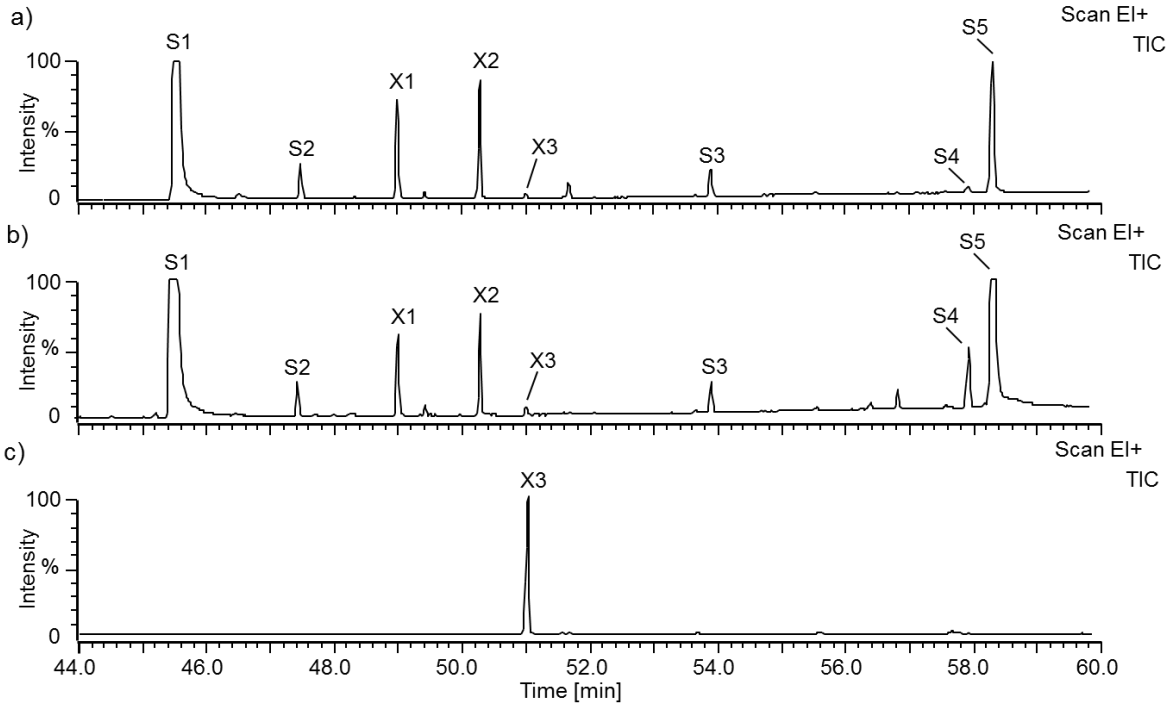


Figure 3. Total ion chromatograms (GC-MS) of methanolic seed extracts recovered from a) *H. perforatum*, b) *H. tetrapterum* and c) synthetic 1,3,6,7-THX as a reference after silylation with BSTFA. Assignment: **S1**, sucrose-TMSi; **S2**-**S4**, saccharide-TMSi derivatives; **S5**, raffinose-TMSi; **X1** and **X2**, THX-TMSi isomers; **X3**, 1,3,6,7-THX-TMSi.

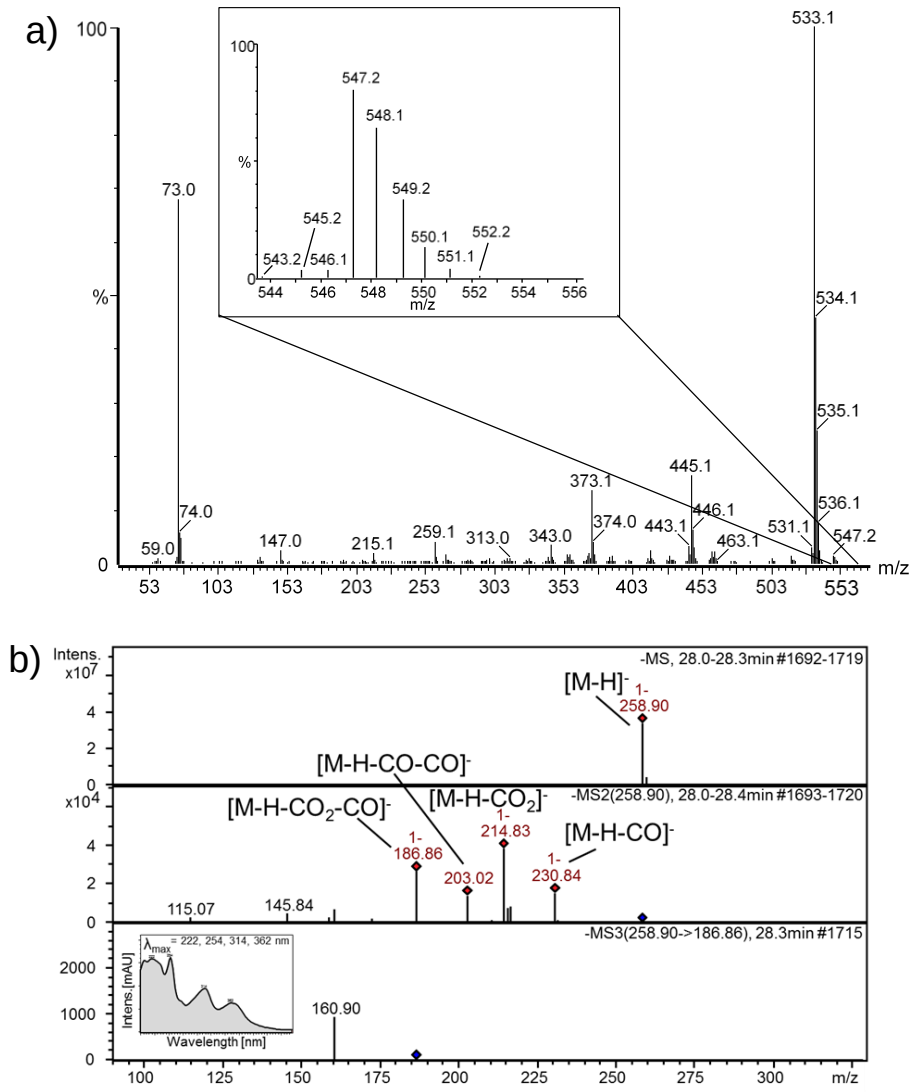
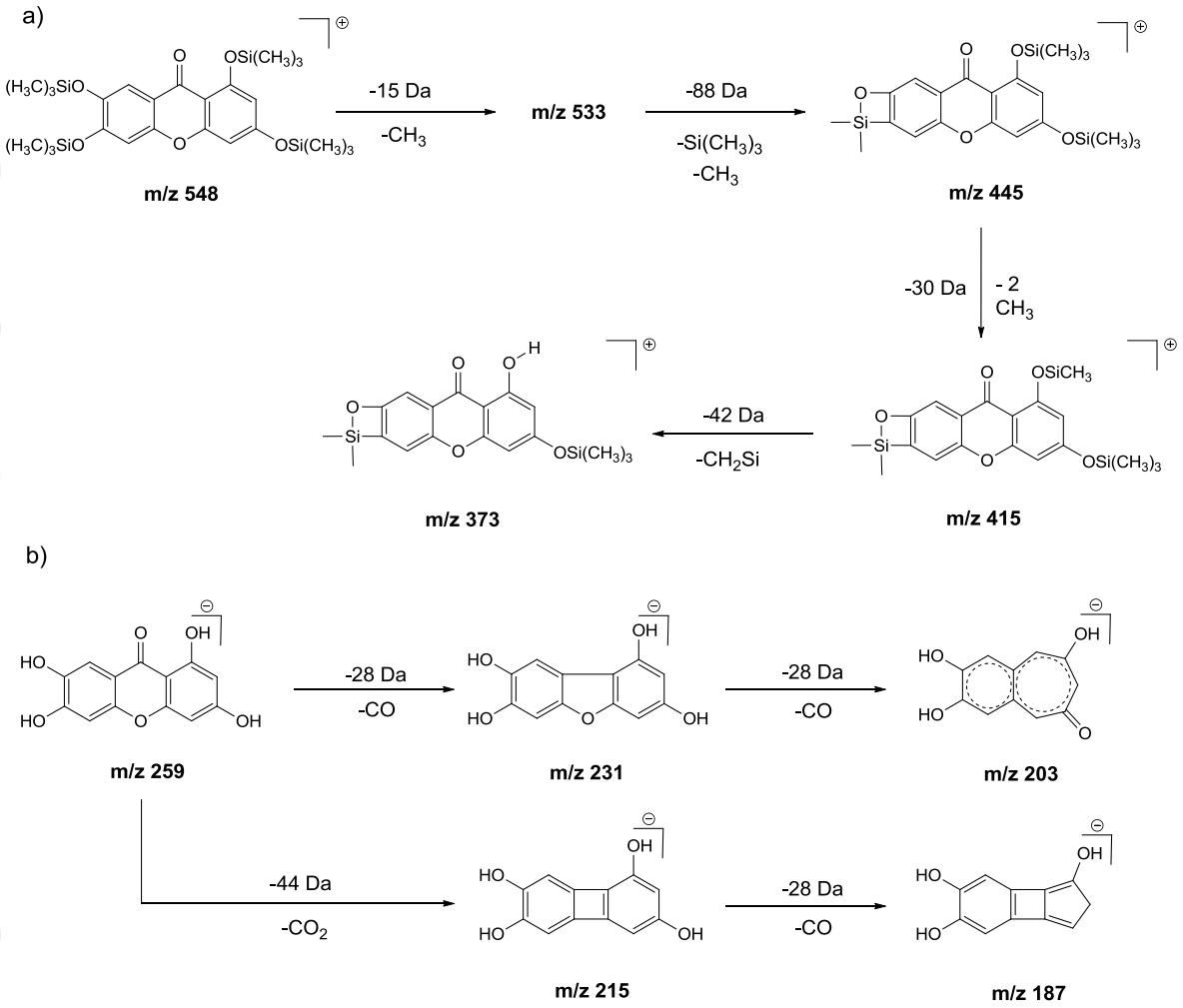


Figure 4. EI- and ESI negative-MS^n mass spectra of compound **X3**. a) **X3**-TMSi ($C_{25}H_{40}O_6Si_4$; M_W 548.92 Da), EI spectrum. The insert exhibits the molecular ion $[M]^+$ at *m/z* 548. b) **X3** ($C_{13}H_8O_6$; M_W 260.20 Da), ESI -MS^n data. The insert exhibits the UV spectrum.



Scheme 1. Proposed MS fragmentation mechanism for 1,3,6,7-THX (**X3**). a) MS fragmentation (EI) for the TMSi derivative, b) MS/MS fragmentation (ESI) corresponding to the mass spectral data of *Figure 4*.

Phenolic constituents of H. perforatum and H. tetrapterum seeds

HPLC-DAD-MS^n analyses were performed for the determination of phenolic seed constituents. Assignment of individual compounds was based on their UV/Vis spectral characteristics and mass spectrometric behavior in negative ionization mode.

Besides **X1**, **X2** and **X3**, which were present as main compounds in both extracts, a range of xanthone derivatives were assigned Table 1. Peak **1** revealed an $[M-H]^-$ ion at *m/z* 329 and a fragment ion at *m/z* 167, thus indicating the loss of an *O*-hexose moiety. Taking together these findings with the UV maxima at 256 and 294 nm, this compound was identified as vanillic acid hexoside [33]. This component was the only phenolic acid hexoside detected in both species. Compound 2 exhibited an $[M-H]^-$ ion at *m/z* 261, which may point to the occurrence of maclurin, being known as a precursor in xanthone biosynthesis [34]. However, spiking experiments with a reference standard proved the extracts to be devoid of maclurin. Consequently, compound **2** was proposed to be an isomer of 3',4,4',6-pentahydroxy-benzophenone (maclurin).

Peaks **3**, **5**, **8** and **10** revealed an $[M-H]^-$ ion at *m/z* 421 as well as product ions at *m/z* 331 ($[M-H-90]^-$) and at *m/z* 301 $[M-H-120]^-$ in MS^2 experiments, thus showing the characteristic loss of a *C*-hexose moiety [32] and a base peak signal at *m/z* 259 , pointing to mangiferin (1,3,6,7-THX-*C*-glucoside). These compounds only differed in their retention behavior and their UV spectra. Astonishingly, spiking experiments with mangiferin as a reference compound unveiled both *Hypericum* extracts to be devoid of mangiferin, since the retention behavior of the latter differed from those of the aforementioned components. Consequently, these compounds were characterized as mangiferin isomers.

Interestingly, although mangiferin was reported to represent the major xanthone-*C*-glycoside in roots of *H. perforatum* [17] and, although mangiferin was found as a major xanthone in cell cultures of *H. perforatum* [35], we did not detect this compound in seeds of both species. However, our findings are in accordance with the studies of Crocket *et al.* (2011) [16], who did not find mangiferin in roots of *H. perforatum* and Dias et al. (2000) [24], who neither detected this compound in *in vitro* biomass of *H. androsaemum*.

Peak **6** also showed an $[M-H]^-$ ion at *m/z* 421, but considerably differed in its fragmentation behavior. It revealed a fragment ion at *m/z* 258 in the MS^2 experiment, indicating the loss of a *O*-hexose moiety and was therefore assigned to THX-*O*-hexoside [32]. Unfortunately, based on the present analytical data it was neither possible to verify the exact position of the glycosylation nor the definite nature of the hexose moiety, but based on MS^2 spectra, a distinction between *O*-glycosides and *C*-glycosides could be made.

An $[M-H]^-$ ion at *m/z* 447 together with the subsequent losses of 90 and 30 Da in the MS^2 spectrum as well as a base peak ion at *m/z* 284 indicated peak 7 to be a trimethoxyxanthone-*C*-hexoside. Peak 20 also revealed an $[M-H]^-$ ion at *m/z* 447, however, the further fragmentation with the loss of 146 Da (loss of a rhamnose moiety) resulted in a base peak fragment ion $[M-H-146]^-$ at *m/z* 301. The compound was assigned to a hydroxy-trimethoxy-xanthone-*O*-rhamnoside. Peaks **9** and **12** exhibited $[M-H]^-$ ions at *m/z* 391 and major fragment ions at *m/z* 331 $[M-H-60]^-$, *m/z* 301 $[M-H-90]^-$, *m/z* 273 $[M-H-90-28]^-$ and at *m/z* 258. It was therefore assigned to a THX-*C*-pentoside. Based on their mass spectrometric behavior, compounds **11**, **13**, **16** and **24** were tentatively identified as *C*-pentosides of trimethoxy-xanthone isomers [36].

Furthermore, according to their spectral data and fragmentation behavior, compounds **4** and **14** were identified as hydroxy-trimethoxyxanthone-*C*-hexoside and hydroxy-trimethoxyxanthone-*O*-hexoside (molecular ions $[M-H]^-$ at *m/z* 463), respectively.

Detailed MS^n data of compounds **31** and **32** are shown in *Figure 5*. Interestingly, compound 32 produced an $[M-H]^-$ ion at *m/z* 485, which revealed a product ion at *m/z* 323 (loss of an *O*-hexose moiety) in the MS^2 experiment and an ion at *m/z* 243 (loss of SO_3) upon further fragmentation [26; 37; 38]. Compound **31** with a molecular ion $[M-H]^-$ ion at *m/z* 323 also showed a characteristic loss of 80 Da, in this case in the MS^2 experiment, resulting in a daughter ion at *m/z* 243. Hence, the compounds were assigned to trihydroxyxanthone-sulfonate-*O*-hexoside and trihydroxyxanthone-sulfonate, respectively. Comparably, peaks **40** and **41** both exhibited an $[M-H]^-$ ion at *m/z* 323, releasing a fragment ion at *m/z* 243 in the MS^2 experiments. The loss of 80 Da was attributed to the release of SO_3, which is in agreement with the tentative assignment to trihydroxyxanthone-sulfonate isomers.

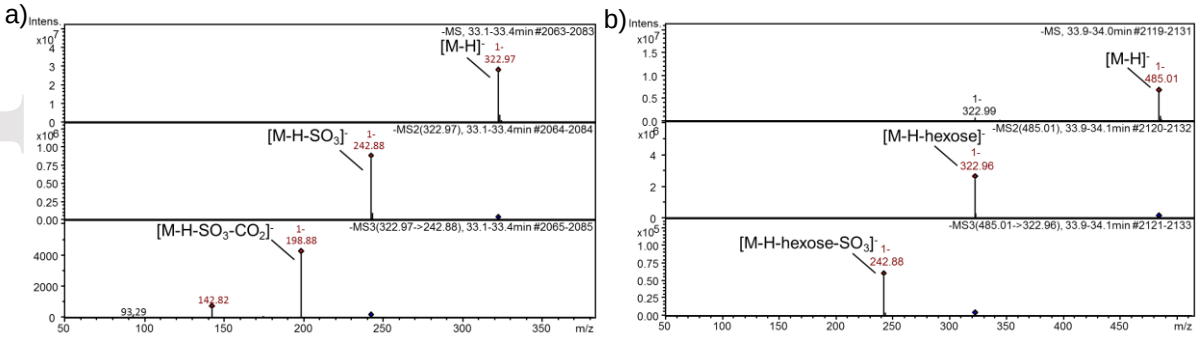


Figure 5. Fragmentation behavior of compound **31** a) and compound **32** b), both recorded applying the negative ionization mode.

Moreover, compounds **23** and **39** both revealed molecular ions $[M-H]^-$ at *m/z* 337, releasing fragment ions at *m/z* 322 and 257, respectively. The loss of 15 Da in the MS^2 spectrum is referred to a methyl radical loss, forming an even product ion at *m/z* 322. Additionally, the neutral loss of 80 Da upon fragmentation indicated the presence of a SO_3 moiety, resulting in a fragment ion at *m/z* 257. The subsequent loss of 15 Da in the MS^3 experiment also indicated the loss of a methyl radical, releasing an even product ion at *m/z* 242. The two compounds were, therefore, tentatively assigned to dihydroxy-methoxyxanthone-sulfonates.

Compound **15** gave a deprotonated molecular ion $[M-H]^-$ at *m/z* 499. The MS^2 spectrum was characterized by the loss of an *O*-hexose moiety (162 Da) resulting in a fragment ion at *m/z* 337. Since the product ions observed in the MS^3 experiment were identical to those of compound **23**, compound **15** was identified as dihydroxy-methoxyxanthone-sulfonate-*O*-hexoside. Up to now, only two natural sulfonated xanthones, *i.e.* 1,3-dihydroxy-5-methoxyxanthone-4-sulfonate and 5-*O*-*β*-D-glucopyranosyl-1,3-dihydroxyxanthone-4-sulfonate, have been reported for *Hypericum sampsonii* [26]. These sulfonated xanthones exhibited significant cytotoxicity against a mouse lymphocytic leukemia cancer cell line [26]. To the best of our knowledge, we herein report the occurrence of sulfonated xanthones in *Hypericum* seeds for the first time. Compounds **26, 27** and **28** exhibited $[M-H]^-$ ions at *m/z* 517 and fragments at *m/z* 259 in MS^2 experiments, which is characteristic of THX dimers [18; 39]. Compounds **29** and **30** showed almost the same absorbance maxima in a range of 220 to 390 nm. Moreover, they predominantly showed doubly deprotonated precursor ions $[M-2H]^{2-}$ at *m/z* 388 and a pseudomolecular ion $[M-H]^-$ of significantly lower abundance at *m/z* 777. Consequently, these compounds were tentatively identified as trimers of THX. Producing an $[M-H]^-$ ion at *m/z* 273, compound **33** was assigned to a trihydroxymethoxyxanthone. Further compounds (**17**, **18**, **19**, **34**, **35**, **36**, **37** and **38**) were determined as xanthone derivatives based on their UV-spectroscopic and mass spectrometric data, but their definite structure could not be further characterized.

Table 1. Assignment of characteristic compounds in methanolic seed extracts of *H. perforatum* and *H. tetrapterum*: HPLC data and mass spectrometric data, recorded in negative ionization mode: precursor ion shows $[M-H]^-$ species.

Peak no.	t_r [min]	Peak assignment[a]	λ_{max} [nm]	MS^n data [*m/z*]			*H. perforatum*	*H. tetrapterum*
				Precursor ion	MS^2	MS^3		
1	9.5	vanilic acid hexoside	214, 256, 294	329.0	*166.7*	*151.7*, 122.9, 107.9	x	x
2	11.8	pentahydroxy-benzophenone	212, 260*sh*, 324	260.8	*230.7*	212.7, *202.7*, 186.7	x	x
3	15.9	mangiferin isomer	210, 256, 316, 364	421.0	330.9, *300.9*, 258.8	272.8, *257.8*	x	x
4	16.5	hydroxy-trimethoxyxanthone-*C*-hexoside	212, 268, 350	463.0	409.0, 373.0, *343.0*	325.0, *315.0*, 300.0	x	*n.d.*
5	16.6	mangiferin isomer	216, 256, 316, 362	421.0	330.9, *300.9*	272.8, *257.8*	*n.d.*	x
6	18.1	tetrahydroxyxanthone-*O*-hexoside	214, 254, 328	421.0	*257.8*	228.7, 185.7, *173.7*, *161.7*	x	x
7	18.2	trimethoxyxanthone-*C*-hexoside	212, 254, 270*sh*, 332	447.0	357.0, *326.9*	*298.9*, 283.9,	x	*n.d.*
8	18.5	mangiferin isomer	218, 252, 278*sh*, 324	421.0	330.9, *300.9*	*272.8*, 257.8	x	x
9	18.6	tetrahydroxyxanthone-*C*-pentoside	218, 256, 316, 362	391.0	331.0, *300.9*	272.8, *257.8*	*n.d.*	x
10	18.8	mangiferin isomer	222, 254, 278, 384	420.9	330.9, 300.9, *257.8*	213.9, 201.7, *185.7*, 157.8	x	x
11	19.6	trimethoxyxanthone-*C*-pentoside	214, 268, 346	417.0	381.0, 357.0, *327.0*	*298.9*, 283.9	x	*n.d.*
12	20.7	tetrahydroxyxanthone-*C*-pentoside	216, 252, 284*sh*, 326	391.0	331.0, *300.9*	*272.8*, 257.8	*n.d.*	x
13	20.8	trimethoxyxanthone-*C*-pentoside	212, 264, 348	417.0	357.0, *326.9*	*298.9*, 283.9	x	*n.d.*
14	21.5	hydroxy-trimethoxyxanthone-*O*-hexoside	216, 256, 360*sh*	463.0	*300.9*	270.8, *178.7*, 150.7	*n.d.*	x
15	21.7	dihydroxy-methoxyxanthone-sulfonate-*O*-hexoside	218, 258*sh*, 312*sh*	498.9	*336.9*	*321.8*, 257.7	*n.d.*	x
16	21.9	trimethoxyxanthone-*C*-pentoside	212, 260*sh*, 346	417.0	356.9, *326.9*	298.9, 283.8	x	*n.d.*
17	22.6	xanthone derivative	216, 266, 328	401.0	341.0, *310.9*	*282.9*, 267.8	x	*n.d.*
18	22.7	xanthone derivative	218, 256, 316, 362	405.0	330.9, *300.9*	272.8, *257.8*	*n.d.*	x
19	23.1	xanthone derivative	218, 254, 276*sh*, 322	565.0	503.0, 463.0, *421.0*	257.8	x	*n.d.*
20	24.0	hydroxy-trimethoxyxanthone-*O*-pentoside	218, 258*sh*, 360	447.0	373.0, *300.9*	270.9, *178.7*	*n.d.*	x
X1	24.8	tetrahydroxyxanthone	204, 222, 256, 282, 320*sh*, 392	258.8	241.8, 228.6	185.7	x	x
X2	25.6	tetrahydroxyxanthone	234, 252, 294, 328*sh*, 392	258.8	228.7	201.7, 172.7	x	x

23	26.4	dihydroxy-methoxyxanthone-sulfonate	222, 248, 302	336.9	*321*.9, 256.8	*257.8,* 241.7	*n.d.*	x
24	26.8	trimethoxyxanthone-*C*-pentoside	210, 258, 348	417.1	357.0, *327.0*	*289.9*, 283.8	x	*n.d.*
X3	28.3	1,3,6,7 tetrahydroxyxanthone	220, 254, 314, 362	258.9	230.8, 214.8*, 203.0*, *186.9*	160.9	x	x
26	30.9	tetrahydroxyxanthone-dimer	218, 258*sh*, 282*sh*, 388*sh*	516.9	498.9, *390.9*, 258.8,	*362.9*, 334.9	x	x
27	31.5	tetrahydroxyxanthone-dimer	220, 260*sh*, 282*sh*, 390*sh*	516.9	498.9, *390.9*, 258.8	*362.9*, 334.9	x	x
28	32.8	tetrahydroxyxanthone-dimer	218, 258, 288, 396*sh*	516.9	498.9, *258.8*	*228.7*, 184.7	x	*n.d.*
29	32.8	tetrahydroxyxanthone-trimer	220, 260*sh*, 290, 390*sh*	388.0[a]	*298.9,* 202.7	270.8, *254.8*	*n.d.*	x
30	33.1	tetrahydroxyxanthone-trimer	220, 258, 290,382*sh*	388.0[a]	*298,8*	270.8, *254.8*, 226.8	*n.d.*	x
31	33.2	trihydroxyxanthone-sulfonate	220, 258, 292, 388*sh*	323.0	*242.9*	*198.9*, 142.8	x	x
32	33.9	trihydroxyxanthone-sulfonate-*O*-glycoside	220, 256*sh*, 292*sh*, 390*sh*	485.0	*323.0*	*242.9*	x	x
33	35.0	trihydroxy-methoxyxanthone	220, 256, 292*sh*, 390*sh*	272.8	*257.7*	*229.7*, 213.6, 172.7	x	*n.d.*
34	36.1	xanthone derivative	220, 258*sh*, 292*sh*, 394*sh*	327.1	309.1, 291.0, 273.0, 247.0, *228.9,* 170.9	*210.8,* 168.7	x	x
35	38.1	xanthone derivative	222, 260*sh*, 290, 324*sh*, 396	775.0	*756.9*, 623.0, 474.9	630.9*, 604.9*	x	*n.d.*
36	38.7	xanthone derivative	220, 260*sh*, 288*sh*, 394*sh*	572.9	528.9, *500.9*	*473.0*, 456.9, 376.9	x	*n.d.*
37	39.8	xanthone derivative	220, 262*sh*, 288*sh*, 392*sh*	329.2	293.0, 228.9, *170.8*	124.8	x	*n.d.*
38	40.8	xanthone derivative	218, 260*sh*, 290*sh*, 392*sh*	459.1	417.0, *236.8*	*176.8*, *161.7*	x	x
39	41.7	dihydroxy-methoxyxanthone-sulfonate	220, 260*sh*, 290, 396*sh*	336.9	*321.9*, 256.8	257.7, 241.7	x	x
40	43.0	trihydroxyxanthone-sulfonate	222, 250, 310	322.9	*242.8*	196.7, 170.7, 153.8	*n.d.*	x
41	44.1	trihydroxyxanthone-sulfonate	222, 310*sh*	322.9	*242.8*	*190*	*n.d.*	x

[a] Precursor ion is due to a $[M-2H]^{2-}$ ion species. Parent ions for the next fragmentation step are presented in italics; *sh* shoulder; *n.d.* not detected.

Antioxidant potential of Hypericum seed extracts and individual phenolic compounds

In the present work, the radical scavenging activity of the methanolic extracts of *H. perforatum* and *H. tetrapterum* was evaluated by means of the DPPH assay. Additionally, the radical scavenging capacity of 1,3,6,7-THX was investigated. The activity of the synthetic antioxidant *Trolox* was used as a reference. The 1,1'-diphenyl-2-picrylhydrazyl (DPPH) assay is widely applied for the determination of the antioxidant activity of either different plant extracts or individual compounds [40]. The assay is based on the reduction of DPPH forming a colorless reaction product in the presence of a hydrogen donating antioxidant [41].

Being in accordance with literature data [42], *Trolox* revealed an IC_{50} value of 6.6 mg/L (*Table 2*). With an IC_{50} value of 8.7 ± 0.23 mg/L, the methanolic extract of *H. perforatum* was found to exhibit similar activity. Interestingly, *H. perforatum* exhibited an approx. 3.5-fold higher radical scavenging capacity as compared to *H. tetrapterum*, which revealed an IC_{50} value of 31.9 ± 0.26 mg/L. These findings can be correlated with the concentrations of THX, which are illustrated in *Figure 6*, since the total THX contents of *H. perforatum* amounted to 1.25 g/kg and of *H. tetrapterum* to 0.27 g/kg, respectively. With an IC_{50} value of 3.0 ± 0.14 mg/L, 1,3,6,7-THX showed the highest antioxidant activity. In summary, these outcomes underline that the contents of total THX are directly proportional to the radical scavenging activity, although other reducing secondary plant metabolites may also be involved in the effects observed.

Table 2. IC_{50} values of methanolic seed extracts of *H. perforatum* and *H. tetrapterum*, *Trolox* and 1,3,6,7-THX in the DPPH assay

No	Test sample	Regression equation[a]	R^2	IC_{50} (mg/L)[b]
1	*Trolox*	y = 7.503 x + 0.4132	0.9960	6.6 ± 0.13
2	*H. perforatum*	y = 5.486 x + 2.101	0.9935	8.7 ± 0.23
3	*H. tetrapterum*	y = 1.536 x + 1.056	0.9956	31.9 ± 0.26
4	1,3,6,7-THX	y = 16.48 x + 0.3449	0.9998	3.0 ± 0.14

[a] y refers to the absorbance measured at 516 nm; x refers to the concentration of the test sample [mg/L]
[b] Means of three separate sample preparations, each measured in duplicate (CV < 5%).

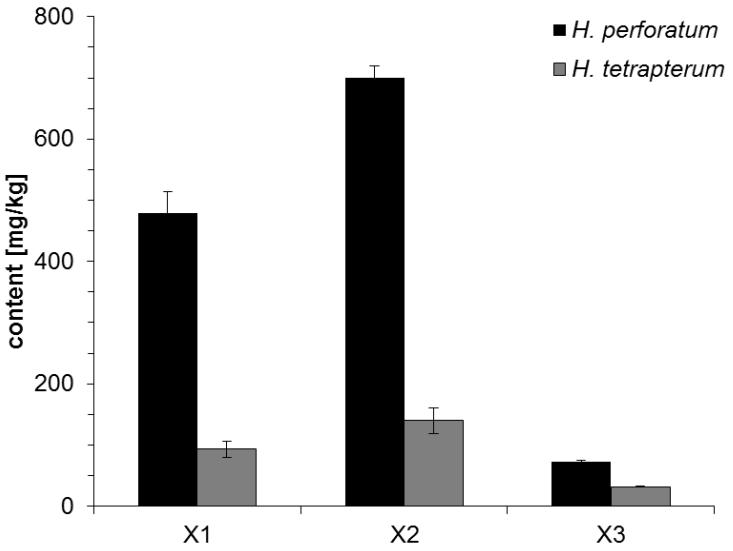


Figure 6. Contents of individual compounds (**X1**, **X2**, **X3**), calculated in mg THX per kg *Hypericum* seeds. Bars represent means ± standard deviation (*n*=3).

Table 3. Validation data for the external quantitation of THX derivatives using a standard solution of 1,3,6,7-THX

Equation of calibration curve[a]	R^2	Precision intraday [%]	Precision interday [%]	Recovery [%]	LOD[b] [µg/mL]	LOQ[c] [µg/mL]
y = 2.532x + 4.402	0.9991	0.91[d]/0.23[e]	0.81[d]/0.30[e]	99.88	0.08	0.27

[a] Detection wavelength λ = 260 nm, [b] Limit of detection calculated on the basis of a signal-to-noise ratio of 3:1; [c] Limit of quantitation calculated on the basis of a signal-to-noise ratio of 10:1; [d] c = 10 µg/mL; [e] c = 100 µg/mL.

Conclusions

Altogether, the present study provides novel information about the phytochemical composition of seeds of *H. perforatum* and *H. tetrapterum*. They are rich in fatty oil, consisting of a series of fatty acids, which are primarily unsaturated and with linoleic acid being the main compound. For the first time, several xanthone derivatives, including sulfonated xanthones, have been reported for seeds of *Hypericum*, while 1,3,6,7-THX was unambiguously identified within this compound class. Marked differences were observed with regard to the profile and contents of xanthone derivatives, however, further analyses applying the methodology presented in the present study are needed to allow a clear-cut chemotaxonomic differentiation of both species based on their secondary metabolite profile. Methanolic seed extracts and 1,3,6,7-THX were investigated with regard to their radical scavenging capacity, showing distinct antioxidant activity in the DPPH assay. The observed endogenous antioxidant activity was attributed to the remarkable content of naturally occurring xanthone derivatives, which were present in both species, whereas amounts were considerably higher in *H. perforatum*. Furthermore, since xanthones were found to constitute an antioxidant principle of protecting plant cells from reactive oxygen species and also function as phytoalexins, helping host cells to impair pathogens [23], we also propose this role for xanthones in *Hypericum* seeds. Due to the presence of xanthones, seeds might be protected against oxidative stress and microbial infestation. Additionally, the results provide a basis for continuative studies in routine investigations, including a higher number of samples, and also focusing on different varieties of *Hypericum* as well as covering diverse cultivation areas, harvest years and dates. Moreover, the outcomes presented in this work could also direct to following investigations with regard to the structural elucidation of further THX, which are under progress. Conclusively, the lipid fraction as well as the phenolic fraction of *Hypericum* seeds might have promising potential in cosmetic and medicinal products.

Experimental Section

General section

Acetonitrile and formic acid of LC-MS grade were purchased from Sigma Aldrich (Steinheim, Germany). Purified water (0.055 µS/cm) was obtained from a Purelab Option-Q system (Elga Berkefeld GmbH, Celle, Germany). The reference standard mangiferin was obtained from Phytolab GmbH (Vestenbergsgreuth, Germany), sucrose and raffinose pentahydrate were obtained from Merck (Darmstadt, Germany). For GC-MS analyses, the silylation reagent N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and the methylation reagent trimethylsulfonium hydroxide (TMSH) were purchased from Fluka (Buchs, Switzerland). For the identification of fatty acids, a fatty acid methyl ester (FAME) reference mixture C16-C24 (Restek Corporation, Bellefonte, PA, USA) was purchased and *tert*-butyl methyl ether (TBME) was obtained from Merck (Darmstadt, Germany). For antioxidant activity testing, 1,1'-diphenyl-2-picryl-hydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (*Trolox*) were obtained from Sigma Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively.

GC-MS analyses

GC-MS analyses of fraction I were carried out according to Lorenz *et al.* (2013) [43] with a Perkin-Elmer 500 gas chromatograph coupled to a single quadrupole mass detector. Injection was performed with a volume of 1 µL and a split ratio of 30:1 at a temperature of 250 °C. A Zebron ZB-5MS cap. column (60 m × 0.25 mm i.d. × 0.25 µm film thickness, Phenomenex, Torrance, USA) was used. The flow rate of the carrier gas (He) was set at 1 mL/min. The temperature profile started at 100 °C, followed by a linear temperature increase of 4 °C/min to 320 °C, with a final hold time of 30 min. The mass spectrometer was run in electron ionization (EI) mode (70 eV). The software Turbomass (v.5.4.2, PerkinElmer Inc., MA, USA) was used for data acquisition and processing. For determination of the fatty acid composition of *H. perforatum* and *H. tetrapterum* seeds, a Varian 450-GC system equipped with a flame ionization detector (FID) and a mass detector (MS-240) (Agilent Technologies Inc., Palo Alto, CA, USA) was used. Aliquots of 1 µL were injected at a temperature of 250 °C. The obtained fatty acid methylesters were separated on a Zebron ZB-WAX-plus capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, Phenomenex, Torrance, CA, USA). The split ratio was 10:1, and the flow rate of the carrier gas (He) was set at 1.4 mL/min. The temperature program of the column oven started with 5 min hold time at 170 °C, subsequently an increase from 170 to 230 °C at 3 °C/min and a final hold time of 20 min at 230 °C was chosen. The mass spectrometer was run in electron ionization (EI) mode (70 eV). The software Varian MS Workstation (v. 6.9.2, Agilent Technologies) was used for data acquisition and processing. Compounds were identified *via* reference standards and by comparison of their mass spectra with those of the NIST spectral database (NIST MS Search, v. 2.0, 2008).

RP-HPLC-(DAD)-ESI-MS^n analyses

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. An XSelect CSH C18 reversed-phase column (100 × 2.1 mm i.d., 3.5 µm particle size; Waters, Wexford, Ireland) was used at 25 °C and a flow rate of 0.34 mL/min. Detection of phenolic compounds was performed at 260 nm. The mobile phase consisted of formic acid/water (0.1/99.9, *v/v*; eluent A) and formic acid/acetonitrile (0.1/99.9, *v/v*; eluent B). The injection volume was 10 µL, and the gradient used was as follows: 0-3 min, 0-5 % B; 3-18 min, 5-20 % B; 18–35 min, 20-30 % B; 35-50 min, 30-55 % B; 50-60 min, 55-100 % B; 60-65 min, 100 % B; 65-70 min, 100-0 % B; 70–75 min, 0 % B. The LC system was coupled to an HCTultra ion trap (Bruker Daltonik GmbH, Bremen, Germany) with an ESI source operating in the negative ionization mode applying the following parameters: capillary voltage: +4000 V, dry gas flow (N_2) 9.00 L/min with a capillary temperature of 365 °C; nebulizer pressure 50 psi. Full scan mass spectra (mass range *m/z* 50-1000) of HPLC eluates were recorded during chromatographic separation. The instruments were controlled by AgilentChemStation B.01.03 (Agilent, Waldbronn, Germany) and esquireControl V 6.1 (Bruker Daltonik GmbH, Bremen, Germany) software. Chromatographic analyses for quantitation purposes were performed on a Dionex Ultimate 3000 RSLC system (Thermo Fisher Scientific GmbH, Dreieich, Germany) equipped with a diode array detector. Chromeleon software (v. 7.1, Dionex, Idstein, Germany) was used for system control and data processing.

NMR spectroscopy

NMR spectra were recorded in DMSO-d_6 at 500 (^{1}H) and 125 MHz (^{13}C), respectively, with a Varian Unity Inova NMR spectrometer (Darmstadt, Germany). Chemical shifts are reported in δ [ppm] and referenced to residual solvent signals of DMSO-d_6 (^{1}H: δ 2.50; ^{13}C: δ 39.51 ppm). ^{1}H- and ^{13}C-NMR signal assignment of compound **X3** is based on increment calculations with a program of *T. Vosegaard* (Version 1.0, Aarhus University, Denmark). For evaluation of NMR spectra, the program SpinWorks 3.1.7. (Copyright 2010, *K. Marat*, University of Manitoba, USA) was used.

Plant material

Three different batches each of seeds of *H. perforatum* and *H. tetrapterum* were purchased from Jelitto perennial seeds GmbH (Schwarmstedt, Germany). Both species were identified by Prof. Dr. O. Spring and voucher specimens (voucher number: HOH-016142 (*H. perforatum*); HOH-016143 (*H. tetrapterum*)) were deposited at the herbarium of the Department of Botany at Hohenheim University (Germany).

Extraction of H. perforatum and H. tetrapterum seeds

100 mL of dichloromethane (CH_2Cl_2) was added to 10 g of *Hypericum* seeds. The suspension was homogenized with an Ultra-Turrax (3 min; 15,000 1/min, IKA Werke GmbH & Co. KG, Staufen, Germany). The first extraction step was performed at 7 °C overnight under the exclusion of light. Afterwards, the plant material was filtered off over *Celite* by vacuum suction, washed with CH_2Cl_2 (50 mL), and subsequently the remaining plant material was re-extracted under the same conditions as described above. The two extracts were combined, and the solvent was removed *in vacuo* yielding fraction I, an oily liquid of 3.06 g (*H. perforatum*) and 1.81 g (*H. tetrapterum*), respectively. A second extraction step was performed with methanol (MeOH). For this purpose, the residue of the plant material was extracted twice with 100 mL of MeOH each at 7 °C overnight. Solvent removal by vacuum rotovaporation yielded 0.32 g (*H. perforatum*) and 0.19 g (*H. tetrapterum*) of fraction II, respectively.

Sample preparation for chromatographic analyses

For comparison with authentic references, mangiferin, 1,3,5,6-THX and 1,3,6,7-THX were dissolved in methanol. For GC-MS analyses, silylation of constituents was performed according to methods previously described in literature [43] to obtain the corresponding trimethylsilyl derivatives. For this purpose, fraction II (6 mg) or purified compounds (1 mg) were dissolved in 0.5 mL of DMF and treated with 300 µL of BSTFA at 105 °C for 15 min and subsequently injected into the GC-MS system. For the determination of retention indices, 50 µL of *n*-alkanes (C28, C29 and C30 in concentrations of 0.2 mg/mL each in chloroform) were utilized [31]. Fatty acid methyl esters (FAME) were prepared by on-column derivatization with trimethylsulfonium hydroxide (TMSH, 0.25 M in methanol). Briefly, 20 µL oil sample was diluted in 2000 µL TBME, 10 µL of the test solution were mixed with 170 µL of TBME followed by 60 µL of TMSH. Consecutively, the mixture was directly injected into the GC-MS system.

Quantitation of tetrahydroxyxanthones (THX) and method validation.

1,3,6,7-THX was used as standard compound for the quantitation of THX isomers. A calibration curve was generated from the signals of six concentration levels (10-100 µg/mL) *via* linear regression analysis by plotting peak areas against sample concentrations. For method validation, intraday precision was determined with standard concentrations of 10 and 100 µg/mL, analyzed six times on the same day. The interday precision was evaluated by analyzing the samples on two different days, respectively. Recovery was calculated using a spiked methanolic solution of fraction II (*H. perforatum*). The resulting concentration was compared with those of the corresponding calibration standard (50 µg/mL) prior to spiking. The HPLC method was proven to be reproducible, reliable and accurate According to the ICH guideline, limits of detection (LOD) and quantitation (LOQ) were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively [44]. For this purpose, the means of eight blank samples were compared with the signal of the lowest calibration sample concentration (10 µg/mL). Associated validation data are presented in *Table 3*.

Stable free radical scavenging activity

The free radical scavenging activities of the methanol seed extracts were determined by the 1,1'-diphenyl-2-picrylhydrazyl (DPPH) assay according to a protocol previously described [43]. Briefly, fraction II (5.0 mg for *H. perforatum* and 10.0 mg for *H. tetrapterum*, respectively) was dissolved in 20 mL of MeOH for the preparation of stock solutions, which were appropriately diluted. 200 µL of a range of concentrations were added each to 1800 µl of DPPH (100 µM) and incubated for 30 min at 37 °C. Subsequently, absorbance ($A_{516\ nm}$) was measured using a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer, Rodgau-

Jüdesheim, Germany) equipped with a UV-Vis software (UV Winlab ES Version 6.0.2). DPPH scavenging activity was expressed as IC_{50} values, which were calculated by plotting $A_{516\ nm}$ against the corresponding concentrations and subsequent regression analyses. Thereby, IC_{50} was defined as the concentrations of the tested compounds (mg/L) required, bringing about a 50% reduction in absorbance referred to the blank. For comparison purposes, 1,3,6,7-THX and *Trolox* were used as a reference antioxidants.

Synthesis of reference compounds

1,3,6,7-THX (**X3**) was synthesized according to a modified literature protocol [45]. Thus, K_2CO_3 (0.421 g, 3.05 mmol) was added to a suspension of maclurin (2,3',4,4',6-pentahydroxy benzophenone, 0.40 g, 1.53 mmol) in 30 mL water under stirring and external ice cooling. Afterwards, the solution was purged with nitrogen for 15 min and $K_3[Fe(CN)_6]$ (1.00 g; 3.05 mmol), dissolved in 30 mL deionized water and de-oxygenated in an analogous manner, was added dropwise, whereby the color turned into dark blue. Then, the reaction mixture was stirred in an ice bath for 5 h, while the temperature was allowed to rise to room temperature. After the addition of water (60 mL) the pH value was adjusted to 3.0 by adding glacial acetic acid and the solution extracted twice with EtOAc (100 mL each). The combined extracts were dried (Na_2SO_4), and the solvent was distilled off by vacuum rotovaporation to yield a crude orange residue (0.66 g). The target product was recovered from the latter by vacuum liquid chromatography (VLC) on silica 60 G (SiO_2, 100 g), preconditioned with CH_2Cl_2. Elution of **X3** was performed with a CH_2Cl_2/MeOH linear gradient (from 100/0 to 85/15, *v/v*), and the fractionation was monitored by TLC. The fractions containing pure **X3** were unified and evaporated to dryness *in vacuo*. Yield: 0.10 g (25.7 % of the theory). Yellowish powder. TLC (SiO_2; $CHCl_3$/AcOH/H_2O, 10/8.4/1.6, *v/v/v*): R_f 0.55 (single spot). GC-MS purity (TMSi derivative): 98.6 %, *m/z* 548 (1, M^+), 533 (100, M-$CH3^+$), 445 (16), 415 (2), 373 (13), 343 (3), 259 (4), 215 (2), 147 (2), 73 (69, $OSi(CH3)3^+$). ^{1}H-NMR (DMSO-d_6, 500 MHz): 13.19 (s, 1H, OH), 7.35 (s, 1H, H-C(8)), 6.83 (s, 1H, H-C(5)), 6.31 (d, ^4J = 1.6 Hz, 1H, H-C(4)), 6.14 (d, ^4J = 1.7 Hz, 1H, H-C(2)). ^{13}C-NMR (DMSO-d_6, 125 MHz): 178.82 (C=O), 164.67 (C(3')), 162.59 (C(3)), 157.30 (C(1)), 154.55 (C(6')), 151.05 (C(6)), 143.90 (C(7)), 111.50 (C(7')), 107.78 (C(8)), 102.52 (C(5)), 101.54 (C(2')), 97.66 (C(4)), 93.58 (C(2)).

1,3,5,6-THX was synthesized according to Guo *et al.* (2015) [46]. The NMR signals were consistent with literature data [46]. The mass spectrum of the TMSi derivative was identical to that of **X3**-TMSi.

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