Analysis of polyphenolic content in marine and aquatic angiosperms from Norwegian coastal waters

Kjersti Hasle Enerstvedi

Thesis for the Degree of Philosophiae Doctor (PhD)
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Dissertation for the degree philosophiae doctor (PhD)

Department of Chemistry

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Preface

This thesis is submitted for the degree of Philosophiae Doctor (PhD) in Chemistry at the University of Bergen, Norway. The experimental part of the work was carried out at the Department of Chemistry, University of Bergen, during the period 2013–2017 under the supervision of Assoc. Professor Monica Jordheim and co-supervisor Professor Øyvind M. Andersen. The thesis consists of three papers (**I**–**III**) preceded by an extended summary.

Chapter 1 gives an introduction to the thesis, chapter 2 presents the methods used in this work, and chapter 3 covers the results from papers **I**–**III**. The Appendix section includes an overview of the compounds in this work (A) and their structures (B), ^{1}H and ^{13}C NMR data (C and D), as well as both 1D and 2D NMR spectra (E and F) for compounds **1**, **3**, **4**, **6**, **9**, **12**, **15**–**17**, **19**–**23** and **CA**. Additional quantitative data for compounds **1**–**12** and **RA** are found in Appendix G, and extraction optimization data are found in Appendix H.

Acknowledgements

First and foremost I would like to thank my supervisors Assoc. Professor Monica Jordheim and Professor Øyvind M. Andersen for excellent supervision. To my main supervisor, Monica Jordheim, I am truly grateful for the guidance and invaluable support you have offered during this work.

I would also like to thank my co-authors, especially Professor Anders Lundberg for sharing your knowledge on marine plants, and for help with collection and identification of botanical material.

I am grateful to The Research Council of Norway for financial support through the Norwegian NMR Platform, and for all support from the technical staff at the Department of Chemistry, especially Assoc. Professor Jarl Underhaug for help with the NMR facilities and Dr. Bjarte Holmelid for recording the high-resolution MS.

Thanks to my colleagues, friends and family for encouragement, support and for making this whole process endurable. Finally I would like to thank my dearest Heine, for being there for me in my darkest times, for making me laugh and for never doubting my abilities.

Abstract

In this work, the polyphenolic content in extracts of *Zostera marina* L., *Zostera noltii* Hornemann, *Ruppia cirrhosa* (Petagna) Grande and *Ruppia maritima* L. from Norwegian coastal waters was characterized for the first time. In *Z. marina* and *Z. noltii* fifteen different flavones, as well as rosmarinic acid were identified. Eight of the flavones were found to be sulphated, among these were luteolin 7,3'-*O*-disulphate and chrysoeriol 7-*O*-sulphate – structures previously not published with complete NMR assignments. In addition, minor amounts of luteolin 7-*O*-β-(6"-*O*-malonyl)glucopyranoside (**6**) and apigenin 7-*O*-β-(6"-*O*-malonyl)glucopyranoside (**11**) were identified in *Z. marina* and *Z. noltii* for the first time. The sulphated flavones were stable in neutral and slightly acidic (< 0.1% trifluoroacetic acid) extracts, but quickly decayed to their corresponding aglycones under more acidic conditions (≥ 0.5% trifluoroacetic acid). Moreover, purified flavonoid sulphates often decomposed during the final steps of isolation, due to increased acid concentrations when the solvents were removed by rotary evaporation. In *R. cirrhosa* and *R. maritima* eight flavonoids were identified, namely the 3-*O*-glucopyranosides and 3-*O*-galactopyranosides, as well as malonylated 3-*O*-glycosides of quercetin and isorhamnetin. The main compound in both species was chicoric acid. None of these compounds have been found in either *Ruppia* species before.

Individual and total phenolic content was quantified in crude extracts of all four seagrass species using analytical HPLC with UV-Vis detection. The flavonoid content was 18.1–24.5 mg/g (DW) in *Z. marina* and 26.2–30.5 mg/g (DW) in most of the examined *Z. noltii* populations. Yet, *Z. noltii* plants collected at the localities Gripnesvågen (C) and Huglo (D), which are in proximity to each other, contained the highest (34.3 mg/g) and lowest (17.3 mg/g) flavonoid concentrations, respectively. The flavonoid content was generally lower in *R. cirrhosa* and *R. maritima* than in the *Zostera* species. However, the phenolic acid content was remarkable high in *Ruppia*, with chicoric acid concentrations in the range of 11.1–12.7 mg/g in *R. cirrhosa* and 27.9–30.2 mg/g in *R. maritima*. The flavonoid content in the three *R. cirrhosa* populations

from different localities on the West coast differed significantly, with flavonoid concentrations ranging from 5.9 mg/g to 14.7 mg/g.

Seasonal variation of both flavonoids and phenolic acids in *Z. marina, Z. noltii* and *R. cirrhosa* was examined. The quantitative variation of flavonoids and rosmarinic acid was found to be relatively consistent from year to year in *Z. marina* during a period of three years. The two *Zostera* species did appear to have a different flavonoid production in the various seasons. While *Z. marina* had the highest content in young leaves in May or June and lowest in February, the opposite was observed in *Z. noltii*, with lowest flavonoid content in May/June and highest in February. The variation of flavonoid content in *R. cirrhosa* appeared to follow a similar pattern as the one observed in *Z. marina*, with the highest concentration of flavonoids in summer (August). However, while the concentrations of rosmarinic acid were highest in late spring/early summer (May/June) in *Z. marina* (3.6 mg/g), peak concentration of chicoric acid was observed in March in *R. cirrhosa* (29.2 mg/g).

The antioxidant activity of *Ruppia cirrhosa* extracts and isolated compounds was investigated spectrophotometrically by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. IC_{50} values were 152.9–175.7 µg/mL for *Ruppia cirrhosa* crude extracts, which is considered low radical scavenging activity. However, a partially purified *R. cirrhosa* extract exhibited very strong radical scavenging activity, with an IC_{50} value of 31.8 ± 0.7 µg/mL. IC_{50} values for isolated flavonoids ranged from 12.1–88.4 µg/mL.

Abbreviations

AP	AcetaminoPhen
CA	Chicoric acid
d_4-MeOD	deuterated methanol
d_6-DMSO	deuterated Dimethyl sulfoxide
DAD	Diode Array Detector
DEPT	Distortionless Enhancement by Polarization Transfer
DQF-COSY	Double Quantum Filter COrrelation SpectroscopY
DW	dry weight
gal	galactose
glc	glucose
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRLC-MS	High Resolution Liquid Chromatography Mass Spectromotry
HSQC	Heteronuclear Single Quantum Coherence
HSQC-TOCSY	Heteronuclear Single Quantum Coherence-Total Correlation SpectroscopY
HSV	Herpes Simplex Virus
Hydro	hydrolittoral
IC_{50}	half maximal Inhibitory Concentration
LOD	Limit of Detection
LOQ	Limit of Quantification
$[M]^+$	Molecular ion
mal	malonyl
NMR	Nuclear Magnetic Resonance
PAPS	3′-PhosohoAdenosine 5′-PhosphoSulphate
RA	Rosmarinic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

RSV	Respiratory Syncytial Virus
SD	Standard Deviation
sub	sublittoral
TFA	Trifluoroacetic Acid
TMS	Tetramethylsilane
TOCSY	Total Correlation SpectroscopY
UV	UltraViolet
UV-Vis	UltraViolet-visible
xyl	xylose

List of publications

I. Enerstvedt, K. H.; Jordheim, M.; Andersen, Ø. M. Isolation and Identification of Flavonoids Found in *Zostera marina* Collected in Norwegian Coastal Waters. *American Journal of Plant Sciences* **2016***, 7,* 1163–1172

II. Enerstvedt, K. H.; Lundberg, A.; Sjøtun, I. K.; Fadnes, P.; Jordheim, M. Characterization and seasonal variation of individual flavonoids in *Zostera marina* and *Zostera noltii* from Norwegian coastal waters. *Biochemical Systematics and Ecology* **2017**, *74*, 42–50

III. Enerstvedt, K. H.; Lundberg, A.; Jordheim, M. Characterization of Polyphenolic Content in the Aquatic Plants *Ruppia cirrhosa* and *Ruppia maritima* —A Source of Nutritional Natural Products. *Molecules* **2018**, *23*, 1–15

1. INTRODUCTION

1.1 Flavonoids

Flavonoids are a large group of polyphenolic compounds found in nature, and more than 8150 different flavonoids have been reported.[1] In plants, flavonoids are reported to function as antioxidants, antimicrobials, visual attractors, photoreceptors, feeding repellant and UV screening.[2-3] Several studies have suggested that flavonoids exhibit biological activities, such as antiviral, anti-inflammatory and cardioprotective effects.[4-7]

1.1.1 Flavonoid structure

Aglycone. The basic flavonoid structure is based on a flavan entity, which consists of a C_6-C_3-C_6 carbon skeleton (Figure 1). There are 12 main subclasses of flavonoids based on the degree of unsaturation and oxidation of the C-ring.[8-9] The most common are flavones, isoflavones, flavonols, flavanones and flavanols (Figure 1). Furthermore, the individual flavonoids within each subclass may be different based on various substitution pattern on the A and B ring. Flavonols and flavones (Figure 2) are two of the major subclasses of flavonoids. Flavones are characterized by the presence of a double bond between C-2 and C-3, and the attachment of the B ring to C-2, whereas flavonols are flavones with a hydroxyl group in the 3-position (3-hydroxyflavones).

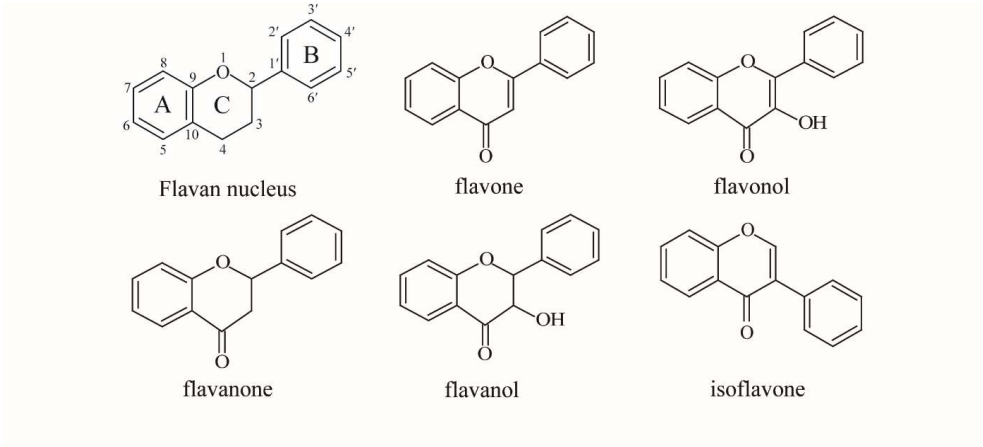
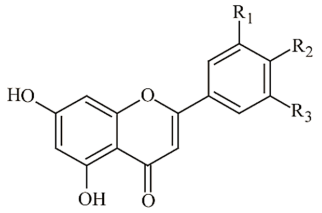
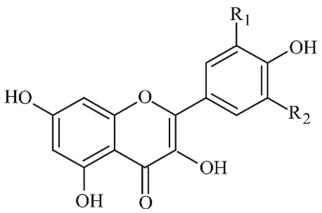


Figure 1. Basic flavonoid structures

Flavonols are widespread in nature, and are mostly found in leaves and external parts of the plant.[9] The most prevalent flavonols in fruits and vegetables are quercetin, kaempferol, isorhamnetin and myricetin.[8, 10] Flavones are less common than flavonols in fruit, but are present in smaller quantities in herbs, grains and leafy vegetables. The most commonly occurring flavones are glycosides of apigenin and luteolin glycosides.[10-11]



	R_1	R_2	R_3
Apigenin	H	OH	H
Luteolin	OH	OH	H
Chrysin	H	H	H
Diosmetin	OH	OCH_3	H



	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Isorhamnetin	OCH_3	H
Myricetin	OH	OH

Figure 2. Structures of common flavones and flavonols

Glycosides. Flavonoids can exist as both free aglycones, but occur commonly as *O*-glycosides. Glycosylation increase the polarity and thus the water solubility of the flavonoid. Most commonly, flavonoids have one or more sugar groups attached in the 3, 5 or 7 position of the aglycone, although sugars have also been found at the other hydroxyl positions.[9, 12-13] On rare occasions, the sugar is directly attached to the aglycone through C-glycosyl linkages.[8] The most common monosaccharide unit found in flavonoids is glucose, followed by galactose, rhamnose, xylose and arabinose (Figure 3), whereas glucuronic and galactoronic acids rarely occur.

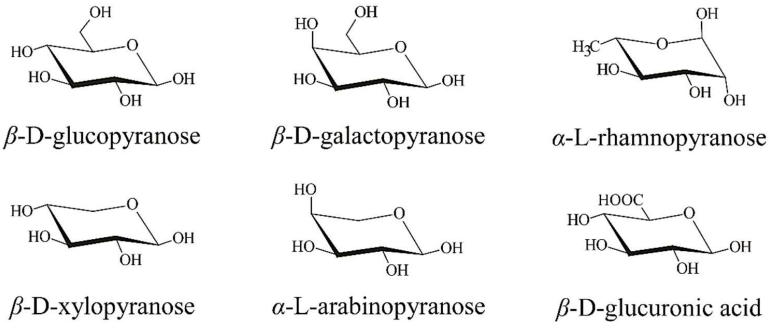


Figure 3. Structures of common monosaccharides found in flavonoids

Acylation. The flavonoids may have one or more aliphatic or aromatic acyl groups attached to the glycoside or direct to a flavonoid hydroxyl. Common aliphatic acids observed in flavonoids are acetic, malonic, lactic, succinic and butyric and quinic acid. Aromatic acids include *p*-hydroxybenzoic, gallic, ferulic and sinapic acids (Figure 4).[8,

13

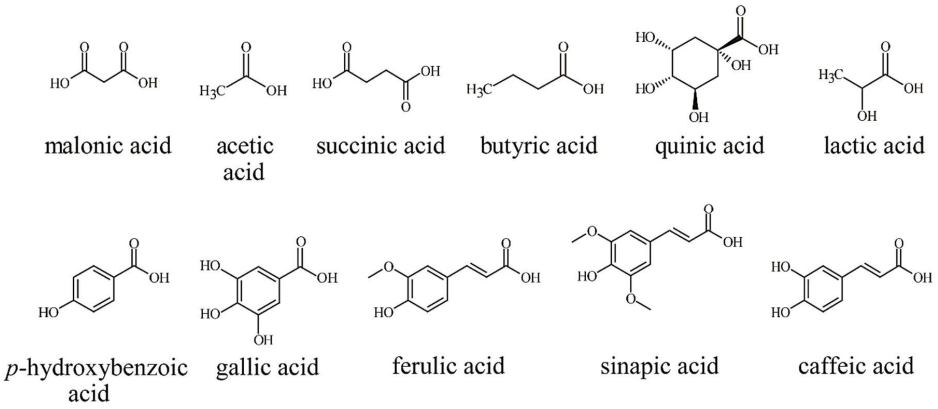


Figure 4. Structures of common aliphatic and aromatic acids found in flavonoids

1.1.2 Biosynthesis

The biosynthesis of flavonoids initiates with condensation of three malonyl coenzyme A molecules with *p*-coumaryl Coenzyme A to form 2′, 4′, 6′, 4-tetrahydroxychalcone (Figure 5), catalyzed by the enzyme *chalcone synthase*. The chalcone is then transformed to the a colourless flavanone by the enzyme *chalcone isomerase.*[14] The flavanone naringenin is an important intermediate, which can be converted to numerous flavonoids by different enzymes. Biosynthesis of flavones usually occurs by direct conversion of flavanones, catalyzed by flavone synthase enzymes (FNSI, FNSII), or via 2-hydroxyflavanones.[15] Flavonols are formed by desaturation of dihydroflavonols, catalyzed by flavonol synthases.[16]

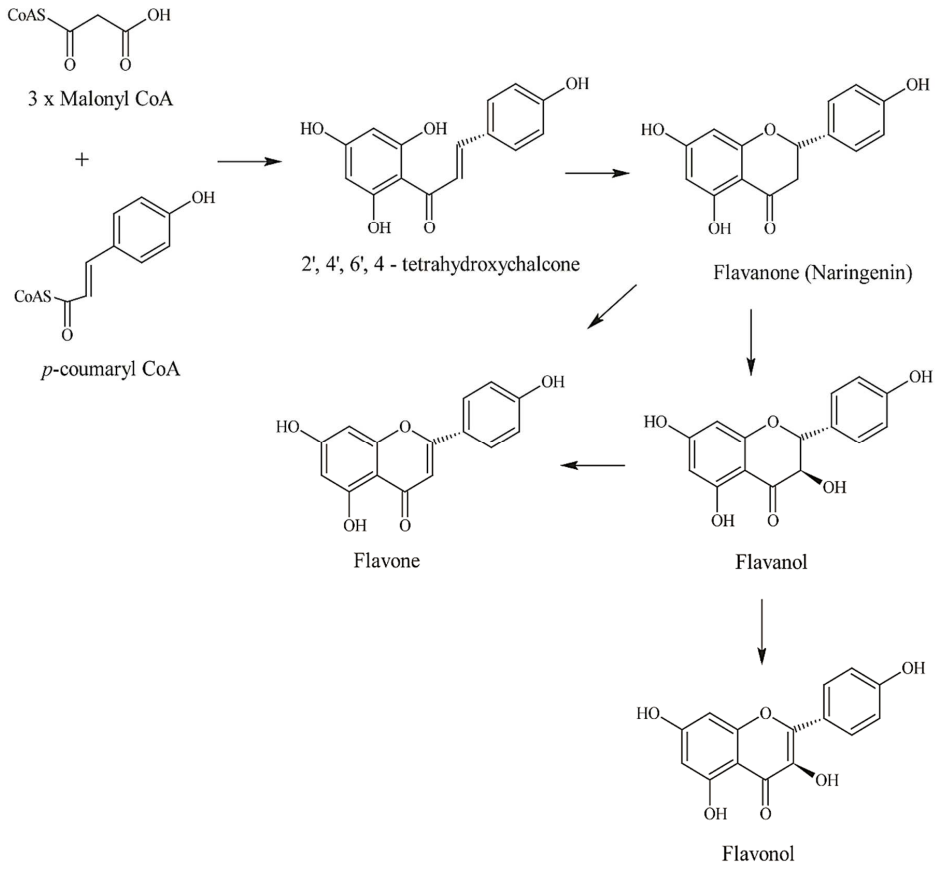


Figure 5. Biosynthetic pathway of flavonoids

1.1.3 Plant function

The functions of flavonoids in plants are mainly associated with protection of the plant, though it has also been suggested that flavonoids are involved in regulation of plant growth and play an important role as signaling molecules.[14, 17-19] Anthocyanins are known to play a significant role in pollination, as they attract both insects and animals with their colours. Some flavones and flavonols also act as insect pollinator attractants.[20] Flavonoids are known to have antibacterial, antifungal and antiviral properties, and increased production of flavonoids in plants has been observed in relation to microbial and fungal attacks, as well as herbivory from insects and mammals.[19, 21-22] As flavonoids have the capacity to absorb UV radiation, they protect the plant from UV-A and UV-B

radiation from the sun. Flavonoids are also considered to have a protecting role against environmental stress, such as extreme (high and low) temperatures and drought.[23]

1.1.4 Biological activities

Antioxidant activity. Flavonoids are commonly known for their wide range biological properties, such as their ability to act as antioxidants. Oxidative stress is thought to be closely linked to various diseases and flavonoids might contribute in disease prevention due to their antioxidant activity.[24-27] The antioxidant capacity is related to the basic flavonoid structure and the substitution pattern. The number of hydroxyl groups, especially on the B ring, is of great importance for the radical scavenging of ROS (reactive oxygen species) and RNS (reactive nitrogen species), as the hydroxyl groups stabilize the reactive radicals by donating hydrogen and electrons to them, thereby generating a relatively stable flavonoid radical.[18, 28] Flavones and catechins are regarded as the most potent flavonoids for protecting the body against ROS.[19, 27] Aglycones usually are stronger antioxidants than their glycosides. *O*-methylation may also reduce the antioxidant activity.[28]

Antibacterial activity. A number of flavonoids, including the widespread flavonoids luteolin, apigenin and quercetin, have been demonstrated to possess antimicrobial and antifungal activity.[19, 29-30] Flavonoids are capable of forming complexes with proteins, and by this means inactivate cell-surface components, enzymes or cell transport proteins of bacteria. Lipophilic flavonoids are also able to interfere with microbial membranes.[18] In general, hydroxylation at position 5 as well as lipophilic substituents at position 6 and 8 on the A ring will improve the antibacterial activity of most flavonoids.[6] Furthermore, hydroxylation at position 3 of the C ring, increase the activity of flavanones.

Antiviral activity. The antiviral activity of flavonoids have been extensively studied. Many naturally occurring flavonoids, including rutin (quercetin 3-*O*-rutinoside), quercetin and kaempferol, exhibit antiviral activity against a number of viruses, such as herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), dengue virus, respiratory syncytial virus (RSV), rhinovirus and human immunodeficiency virus (HIV).[18, 31-34] The antiviral

activity is a result of the inhibition of different enzymes related to the life cycle of viruses.

Anti-Inflammatory activity. Inflammation is a biological response to injury, microbial infection and irritation of body tissue. The purpose is to remove the cause of injury and start tissue healing. Normally, the inflammation is a short term and self-limiting protective response. If the inflammation response is prolonged, it can lead to numerous chronic diseases like diabetes, cancer, cardiovascular and neurodegenerative diseases.[18, 35-37] Many flavonoids, such as apigenin, luteolin, kaempferol and quercetin, are reported to possess anti-inflammatory and analgesic effects,[37-38] in that way contributing to the inhibition of the inflammation process and improving the immune system.

Hepaprotective activity. Chronic diseases such as diabetes, or drug and alcohol abuse may lead to liver damage. Individual flavonoids, including luteolin and quercetin, as well as flavonoid rich plant extracts have been observed to reduce carbon tetrachloride (CCl_4) or acetaminophen (AP) induced hepatotoxicity.[39-41]

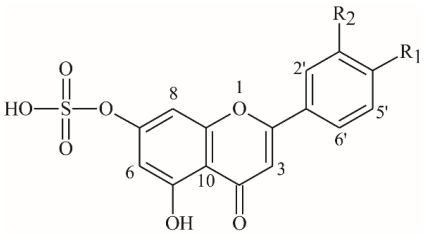
Anticancer activity. Cancer is a major health problem and one of the leading causes of mortality globally. A diet based on a high intake of fruits and vegetables is associated with a lowered risk for developing cancer. This chemo preventive effect is related to the flavonoid content in these foods. Flavonoids are thought to inhibit cancer cell growth, and are considered to be involved in different mechanism, such as carcinogen inactivation, anti-proliferation, cell cycle arrest, induction of atoptosis, and inhibition of angiogenesis, by interacting with various genes and enzymes.[19, 27-28, 42-43] The anticancer activity of flavonoids is influenced by their chemical structure and concentration.[44] Generally, anticancer activities of the metabolites, phenolic acids and aglycones, are higher than those of glycosides.[45] As cancer cells from different body tissue show different sensitivity towards flavonoids, the type of cancer will also affect the cytotoxicity of flavonoids. For instance, luteolin and kaempferol have been proposed as potential anticancer agents for gastric and ovarian cancers, respectively, whereas apigenin, chrysin and luteolin have shown anticancer activity against cervical cancer.[44]

1.1.5 Sulphated flavonoids

Since the first reported sulphated flavonoid isorhamnetin 3-sulphate was isolated from *Polygonum hydropiper* L. (Polygonaceae) in 1937,[46-47] more than 150 sulphated flavonoids have been found in a number of higher plants, including seagrasses.[47] Most flavonoid sulphates are based on hydroxyflavones or hydroxyflavonols, and the sulphate ester is usually linked directly to the aglycone (*O*-sulphates) (Figure 6), and occasionally to the 3 or 6 position of sugar in flavonoid glycosides.[48] Flavone sulphates are usually based on apigenin and luteolin derivatives.[46, 49]

Sulphate flavonoids are formed by a substitution reaction between the flavonoid and the sulphate donor 3'-phosphoadenosine 5'-phosphosulphate (PAPS). The transfer of the sulphonate group, SO_3^-, of the sulphate donor to hydroxyl groups in the flavonoid is catalyzed by sulfotransferases.[17]

Sulphation has generally been considered as a detoxification pathway, as sulphation increases polarity and water solubility, thus facilitating elimination from the body.[47]
In plants, sulphated flavonoids are reported to be involved in plant growth regulation,[17, 50-51] and they are able to form stable complexes with other flavonoids, such as anthocyanins.[51] It has also been suggested that sulphation of flavonoids represents an ecological adaptation for plants growing in saline environment, due to the presence of sulphated flavonoids in numerous plants growing in marine habitats.[4, 17] Flavonoids are in general known for their wide range of biological activities, as described in section 1.1.4. Several studies have addressed in particular sulphated flavonoids for their anticoagulant,[17, 50] anti-inflammatory, antiviral and antitumor activities.[51]



	R_1	**R_2**
Apigenin 7-*O*-sulphate	OH	H
Luteolin 7-*O*-sulphate	OH	OH
Diosmetin 7-*O*-sulphate	OCH_3	OH

Figure 6. Structures of common flavonoid sulphates

1.2 Phenolic acids

Phenolic acids are the most widely distributed secondary metabolites in plants, frequently found in a wide variety of nuts, fruits, berries and roots.

1.2.1 Structure

Usually, phenolic acids are divided into two groups: hydroxybenzoic acid derivatives, containing seven carbons (C6-C1) and hydroxycinnamic acid derivatives, comprising nine carbons (C6-C3).[52-54] Hydroxybenzoic acids may be present in a soluble form conjugated with sugars or organic acids as well as bound to cell wall fractions, such as lignin.[55-56] The most common hydroxybenzoic acids are *p*-hydroxybenzoic acid, vanillic, syringic and protocatechuic acid (Figure 7a).[54-55] Hydroxycinnamic acids are more common than hydroxybenzoic acids, and usually occur as *O*-glyosides or esters of hydroxyacids, such as quinic, shikimic and tartaric acids, whereas they rarely are found in free form.[52, 56] The most widely naturally occuring hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic and sinapic acids (Figure 7b), along with chlorogenic acid, which is caffeic acid esterified with quinic acid.[52, 54-57]

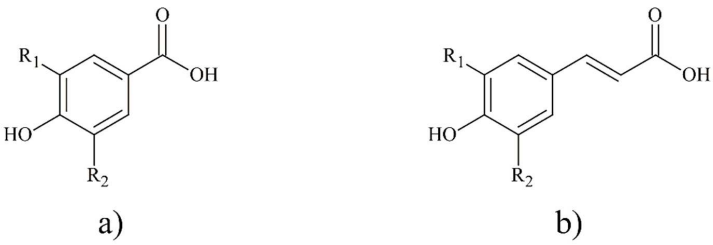


Figure 7. Chemical structures of **a)** *p*-hydroxybenzoic acid (R_1 = H, R_2 = H), protocatechuic acid (R_1 = H, R_2 = OH), vanillic acid (R_1 = H, R_2 = OCH_3) and syringic acid (R_1 = OCH_3, R_2 = OCH_3); **b)** *p*-coumaric acid (R_1 = R_2 = H), caffeic acid (R_1 = OH, R_2 = H), ferulic acid (R_1 = OCH_3, R_2 = H)

1.2.2 Biosynthesis

Most phenolic acids are produced in plants from L-phenylalanine or L-tyrosine via the shikimate pathway.[53] Deamination of the amino acids give rise to cinnamic and/or *p*-

coumaric acids, which may be transformed to various phenolic acids through different hydroxylation and methylation reactions.

1.2.3 Plant function and biological activity

As in the case of flavonoids, phenolic acids act as signaling molecules and growth regulators, and play a major role in plant defense against pathogens and environmental stress factors.[53, 58] They are also reported to possess important biological and pharmacological properties, such as antioxidant,[59] anti-microbial, anti-viral, anti-inflammatory, and anticarcinogenic activities.[55, 59-60]

1.3 Flavonoids and phenolic acids in marine angiosperms

1.3.1 Seagrasses

Seagrasses are marine flowering plants and are considered to be derived from land plants which have recolonized marine habitats.[22, 61] Accordingly, seagrasses have some similarities to vascular land plants concerning their primary and secondary metabolism.

Seagrasses belong to the order Alismatales, and are assigned to four different families Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae.[22] Worldwide, there are more than 70 species of seagrasses, but only five species have been found in European waters; namely *Zostera marina* Linnaeus (eelgrass), *Zostera angustifolia* (Hornemann) Reichenbach (narrow-leaved eelgrass), *Zostera noltii* Hornemann (dwarf eelgrass), *Cymodocea nodosa* Ucria (little Neptune grass) and *Posidonia oceanica* Linnaeus (Neptune grass).[62] Three of these, *Z. marina* (Figure 8a), *Z. angustifolia* and *Z. noltii* (Figure 8b), are found in Norwegian coastal waters.

The two aquatic species *Ruppia cirrhosa* (also known as *Ruppia spiralis*) and *Ruppia maritima* (Figure 8c), belonging to the widgeon grass family, are also native to Norwegian waters. Even though *Ruppia* is not considered to be a true marine plant, it has been included in the Cymodoceae family,[63] and have ecological resemblances to other seagrasses. *Ruppia* species usually occur in brackish or saline waters in temperate

and tropical regions, but are also found in diluted fresh water or fresh water with high salinity, and in a few cases under marine conditions.[64-66]

Most seagrasses exhibit a mixture of clonal growth, i.e. vegetative growth through rhizome extension, and sexual reproduction, though clonal growth is of greatest importance, as sexual reproduction is dependent of the pollen to reach stigmas.[67]

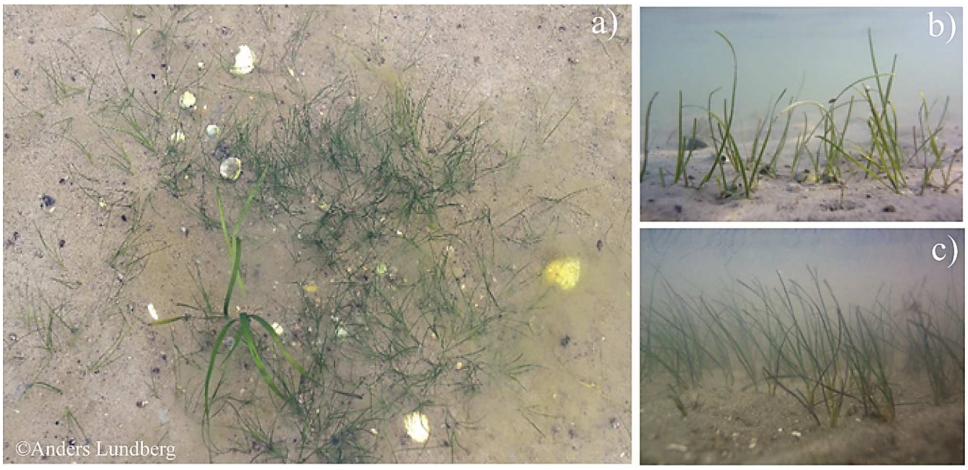
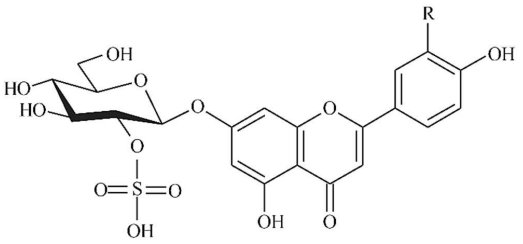


Figure 8. **a)** Two single *Z. marina* plant in the midst of a small *Z. noltii* population.; **b)** *Z. noltii*; **c)** *R. maritima*. Photos by Anders Lundberg

1.3.2 Flavonoids in seagrasses

The flavonoid content in seagrasses varies widely between different genera and species (Table 1). Flavones are predominantly found in seagrass belonging to the families Hydrocharitaceae (*Thalassia*, *Halophila* and *Enhalus*) and Zosteraceae *(Zostera* and *Phyllospadix*), whereas flavonols are mainly found in Posidoniaceae and Cymodoceaceae (*Cymodocea* and *Thalassodendron*). The most frequently occurring flavones in seagrasses are apigenin, luteolin, chrysoeriol, as well as their glycosylated and sulphated derivatives, including thalassiolin A, B and C (Figure 9). Some of the most common flavonols, namely quercetin and isorhamnetin (Figure 2), have been identified in both *Posidonia oceanica* and *Cymodocea nodosa*.[68-70] In *C. nodosa*, the flavonols occur as glycosides, while in *P. oceanica* only aglycones are found. Sulphated

flavones have been found in species of Zosteraceae (*Z. marina and Z. noltii*) and *Thalassia* (*T. hemprichii* and *T. testudium*). In addition, McMillan et al.[71] reported on the presence of sulphated flavones in *Phyllospadix*, *Enhalus* and *Halophil*a, based on an electrophoretic survey of 43 different seagrass species, however, the structure of these flavones were not revealed.



	R
Thalassiolin A	OH
Thalassiolin B	OCH_3
Thalassiolin C	H

Figure 9. Structures of thalassiolin A, B and C.

1.3.3 Phenolic acids in seagrasses

Phenolic acids are widespread in all genera of seagrasses (Table 2). Caffeic acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric and protocatechuic acid are the most predominantly phenolic acids in seagrasses. The seagrass *Posionia oceanica* differ somewhat from the other seagrass species by the extended number of phenolic acids, with a total of 17 different phenolic acids have been detected in this species. These results have been questioned as they are based on paper chromatography and harsh extraction procedures, likely to result in hydrolysis and artefact formation.[22]

1.3.4 Quantitative measurements

Flavonoids. Several authors have reported on total flavonoid content in different seagrasses, mostly in tropical places.[72-77] The quantitative flavonoid content varies greatly between different seagrass species, but ranges from 0.07–5.12 mg/g in most examined species. Even within the same species, the variations can be considerable, exemplified by the flavonoid content in *C. serrulata* and *C. rotundata*, which is in the range of 0.16–5.12 and 0.30–4.56 mg/g, respectively.[73-74, 76] The results in the above

mentioned studies are all based on determination of total flavonoid content using the aluminium chloride colorimetric method, and amounts of individual flavonoids have not been considered. In Europe, only a few seagrass species are known, and merely three different species have been examined for their flavonoid content. The flavonoid content in *Posidonia oceanica* ranges from 0.44–0.52 mg/g, based on the flavonols myricetin, quercetin, isorhamnetin and kaempferol.[78] The flavonoid content in the two *Zostera* species *Z. marina* and *Z. noltii* is considerably higher. In a study performed by Grignon-Dubois, the flavonoid content in *Z. noltii* was reported to range from 3.378–9.895 mg/g.[79] In a different study by the same group the reported quantitative amounts for *Z. marina* (23.7–42.8 mg/g) and *Z. noltii* (52.2–89.2 mg/g) were substantially higher,[80] though it is not clear whether the data are expressed as mg per g dried extract or per g plant material. In a more recent and comprehensive study, where *Z. noltii* samples collected from fifteen different study sites were analysed, the flavonoid content ranged from 7.13–25.06 mg/g.[81] In the latter studies, individual flavonoids were quantified using analytical HPLC with UV-Vis detection.

Phenolic acids. The phenolic content of seagrasses varies a lot between different species, but regional differences within the same species are also seen. The amount of phenolic acids is generally lower in *P. oceanica* than in other seagrass species, with concentrations ranging from 0.314 mg/g in young leaves to 0.451 mg/g in mature leaves. In *Zostera* species, the total phenolic acid content is 13.3–19.2 mg/g for *Z. marina*,[82-83] whereas the concentrations ranged from 0.933 mg/g to 12.094 mg/g for *Z. noltii* from different localities.[84] Main phenolic acids in both *Z. marina* and *Z. noltii* is rosmarinic acid, followed by caffeic and zosteric acid. In *C. nodosa* and *S. filiforme*, chicoric acid (**CA**) is the main phenolic acid, with concentrations of 8.143–27.44 mg/g[69] and 0.94–5.26 mg/g,[85] respectively.

1.3.5 Seasonal fluctuation

Seasonal variations in flavonoid and phenolic concentrations have been observed in a number of terrestrial plants.[23, 86-91] The production of secondary metabolites, including flavonoids and phenolic acids, is influenced by abiotic and biotic environmental factors, as well as the annual cycle of the plant. In some plants, the flavonoid content can be

significantly higher in summer than in the other seasons,[23, 88] which is commonly explained by the increased UV radiation in summertime.

The biomass and production of seagrasses may vary from year to year and throughout the season, depending on the species and latitude. Whereas only little fluctuation occurs in biomass of subtropical/tropical seagrasses, some seagrasses in temperate regions, like *Z. marina*, disappear during winter and grow up from rhizomes and seeds in spring.[92]

Flavonoids. The number of studies investigating seasonal variation of flavonoids in seagrasses is limited. Grignon-Dubois and Rezzonico[79] revealed a decrease in flavonoid content in October compared to June for *Z. noltii* sampled in Spain and France. A seasonal variation of flavonoids was also observed in the seagrass *Thalassia testudinum* outside Cuba, where the total flavonoid content ranged from 9.47 mg/g (January) to 51.30 mg/g (November).[72]

Phenolic acids. The seasonal variation of phenolic acids in seagrasses has been examined in several studies,[93] although the seasonal pattern is not entirely clear. According to Ravn et al.[83] the phenolic concentration (rosmarinic acid and caffeic acid) in *Z. marina* was highest in spring, and low in summer and autumn, whereas Achamlale et al.[94] reported of peak concentration of zosteric acid in summer and autumn in *Z. marina*, and in winter in *Z. noltii*. In *Z. marina* the phenolic concentration correlated with increased light intensity and lower temperatures, as well as with bacterial infection.[82, 95] The concentration of chicoric acid was highest in young leaves of *P. oceanica*, yet the total phenolic content was slightly higher in the mature leaves.[93]

Table 1. Flavonoids found in seagrasses

Family	**Hydrocharitaceae**					**Cymodoceaceae**		**Posidoniaceae**	**Zosteraceae**			
Genera	*Enhalus*	*Halophila*		*Thalassia*		*Cymodocea*	*Thalassodendron*	*Posidonia*	*Zostera*		*Phyllospadix*	
Species	*Enhalus acoroides*	*Halophila stipulacea*	*Halophila johnsonii*	*Thalassia hemprichii*	*Thalassia testudinum*	*Cymodocea. nodosa*	*Thalassodendron ciliatum*	*Posidonia oceanica*	*Zostera marina*	*Zostera noltii*	*P. iwatensis*	*P. japonica*
Acacetin 5-methylether												+ [96]
Apigenin	+ [97]	+ [98]	+ [99]		+ [77]					+ [79]		
4'-glc		+ [100]										
7-glc		+ [100]			+ [77]					+ [79]		
7-(6''-mal)glc		+ [100]										
7-sulphate									+ [101]	+ [79]		
Asobetin							+ [102-103]					
asebogenin 6-rut							+ [103]					
3-hydroxyasobetin							+ [102]					
Catechin							+ [102]					
chrysoeriol		+ [98]			+ [77]							
4'-glc		+ [100]										
7-glc		+ [100]			+ [77]							
7-sulphate					+ [101]				+ [101]			
Diosmetin									+ [104]	+ [79, 104]		
7-glc									+ [104]	+ [104]		
7-sulphate									+ [101]	+ [79, 101]		
Genkwanin		+ [98]										
4'-glc		+ [100]										
4'-(6''-mal)glc		+ [100]										
Hispidulin											+ [105]	+ [96]
Hispidulin acetate												+ [96]

	Enhalus acoroides	*Halophila stipulacea*	*Halophila johnsonii*	*Thalassia hemprichii*	*Thalassia testudinum*	*Cymodocea nodosa*	*Thalassodendron ciliatum*	*Posidonia oceanica*	*Zostera marina*	*Zostera noltii*	*P. iwatensis*	*P. japonica*
Isorhamnetin								+ [68, 106]				
3-glc						+ [69]						
3-rut						+ [69]						
3-(peracetyl)glc						+ [70]						
Isoscutellarein				+ [107]								
7-xyl				+ [107]								
7-(2''-*O*-sulphate)xyl				+ [107]								
Jaceosidin												+ [96]
7-glc			+ [99]									
7-(6-acetyl)glc			+ [99]									
Kaempferol			+ [99]					+ [68, 106]				
Ladanetin			+ [99]									
7-glc			+ [99]									
7 -(6-acetyl)glc			+ [99]									
7-(6-coumaryl)glc			+ [99]									
7-(6-caffeoyl)glc			+ [99]									
Luteolin	+ [97]		+ [99]							+ [79]	+ [105]	
acetate												+ [96]
5,4'-dimethylether												+ [96]
7,3'-disulphate									+ [101, 108]			
3'-glucoronide	+ [97]											
4'-glucoronide	+ [97]											
7-glc									+ [104]	+ [104]		
5-methylether												+ [96]
7-sulphate									+ [101, 108]	+ [79, 101]		
3'-sulphate					+ [77]							
Myricetin			+ [99]					+ [68, 106]				

	Enhalus acoroides	*Halophila stipulacea*	*Halophila johnsonii*	*Thalassia hemprichii*	*Thalassia testudinum*	*Cymodocea nodosa*	*Thalassodendron ciliatum*	*Posidonia oceanica*	*Zostera marina*	*Zostera noltii*	*P. iwatensis*	*P. japonica*
Pedalitin			+ [99]									
7-glc			+ [99]									
7-(6''-acetyl)glc			+ [99]									
7-(6''-coumaryl)glc			+ [99]									
Phloretin								+ [68]				
Phloridzin								+ [68]				
Phyllospadin											+ [105]	
Quercetin								+ [68, 106]				
3-glc						+ [69]						
3-rut						+ [69]						
3-(peracetyl)glc						+ [70]						
3-xyl							+ [102]					
3,7-diglc							+ [103]					
Pectlinargigenin												+ [96]
Rutin						+ [69]						
Scutellarein 7-glc			+ [99]									
Spicoside			+ [99]									
Thallassiolin A				+ [109]	+ [110-111]							
Thallassiolin B				+ [109]	+ [77, 110-111]							
Thallassiolin C					+ [77, 110-111]							
6-hydroxyluteolin												+ [96]
5,7-dihydroxy-3',4'-dimethoxyflavone 7-glc					+ [77]							
5-hydroxy-7,3',4'-trimethoxyflavone				+ [109]								
4'-hydroxy-5,7, 3'-trimethoxyflavone				+ [109]								
5,7,3-trihydroxy-6,4-dimethoxyflavone												+ [96]

[1] glc = glucoside, rut = rutinoside, xyl = xyloside, mal = malonyl

Table 2. Phenolic acids in seagrasses

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	Ref.
Hydrocharitaceae																							
Enhalus																							
E. acoroides												+	+		+	+	+						112-113
Halophila																							
H.engelmannii			+									+			+	+	+				+		113
H. minor			+										+		+	+							113
H. hawaiiana			+											+	+	+	+				+		112-113
H. ovalis			+									+	+		+	+	+						112-113
Thalassia																							
T. hemprichii			+		+							+	+		+	+	+				+		107, 109, 112-113
T.testudinum	+		+									+		+	+	+	+				+		77, 111, 113-115
Potamogetonaceae																							
Cymodoceaceae																							
Cymodocea																							
C. nodosa				+	+			+	+														69
C. rotundata			+									+	+	+	+	+	+				+		113
C. serrulata			+									+	+	+	+	+	+				+		113
Halodule																							
H. uninervis			+									+	+	+	+	+	+				+		113
H. wrightii			+									+		+	+	+	+				+		113
Syringodium																							
S. isoetifolium			+									+	+			+	+				+		113
S. filiforme			+	+	+		+					+			+	+	+			+	+		85, 113

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Ref.
Thalassodendron																							
T. ciliatum			+									+	+	+	+	+	+				+		102-103, 113
Posidoniaceae																							
P. oceanica		+	+	+	+		+		+	+	+	+	+	+	+	+	+		+	+	+		68, 70, 93, 112-113, 116-117
Ruppiaceae																							
R. maritima																		+					94
Zosteraceae																							
Z. marina		+	+			+						+	+	+	+	+	+	+			+	+	94, 104, 112-113 118-119
Z. noltii			+			+						+	+		+	+		+				+	79, 94, 104, 112-113
Z. muelleri			+												+	+							113
Z.capricorni			+									+	+	+	+	+	+						113
Phyllospadix																							
P. scoulerii			+									+	+	+	+	+	+				+		113
P. torreyi			+												+	+							113
P. serrulata			+										+	+	+	+	+				+		113
Heterozostera																							
H. tasmanica																+							113
Amphibolis																							
A. antarctica			+									+	+		+	+	+				+		113
A. griffithii			+									+			+	+	+				+		113

[1] **1** = 3,4-dihydroxybenzoic acid, **2** = benzoic acid, **3** = caffeic acid, **4** = caftaric acid, **5** = chicoric acid, **6** = chlorogenic acid, **7** = cinnamic acid, **8** = coutaric acid, **9** = di-coumaroyl-tartaric acid, **10** = di-feroyl-tartaric acid, **11** = fertaric acid, **12** = ferulic acid, **13** = gallic acid, **14** =gentisic acid, **15** = *p*-coumaric acid, **16** = *p*-hydroxybezoic acid, **17** = protocatechuic acid, **18** =rosmarinic acid, **19** = sinapic acid, **20** = syringic acid, **21** = vanillic acid, **22** = zosteric acid

2. METHODS

The experimental procedures and analytical methods used in this work are described in five sections: plant sampling (2.1), extraction and purification (2.2), separation and isolation (2.3), analytical methods (2.4) and antioxidant activity (2.5). Additional experimental details can be found in the individual papers (**I**–**III**).

2.1 Plant sampling

Plant material was collected during spring low tide by hand from twelve different study sites in the southern coast of Norway (Table 3, Figure 10). The collected plant material was washed thoroughly in fresh water and air-dried. The root was separated from the rest of the plant, and the material was cut in small pieces and stored at –20 °C, when not used.

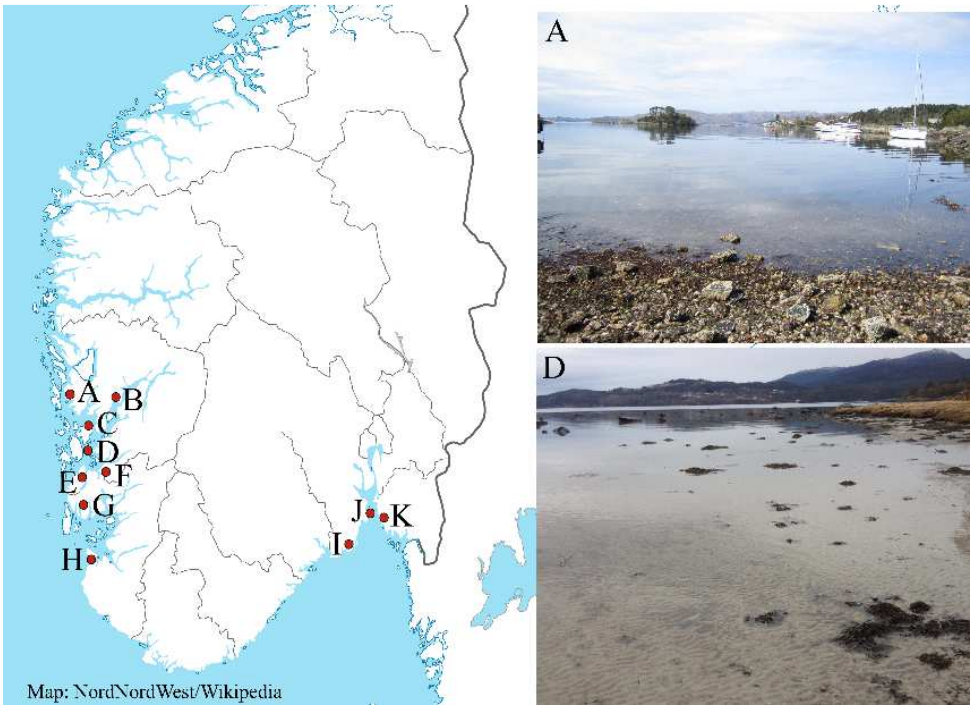


Figure 10. Left: Sampling sites (A-K) for *Zostera* and *Ruppia* samples along the southern coast of Norway. Top right: Espegrend location (A); Below: Huglo location (D)

Table 3. Sample localities of *Zostera marina*, *Zostera noltii*, *Ruppia cirrhosa* and *Ruppia maritima* in the southern part of Norway

	Locality	County, municipality	Coordinates	Zone[1]	Depth (cm)	Plant collected
A	Espegrend	Hordaland, Bergen	60°16'12.0"N, 05°13'20.3"E	sub	40–100	*Z. marina*
	Røytepøyla	Hordaland, Bergen	60°15′34.5"N, 05°15′57.9"E	sub	20–100	*R. cirrhosa*
B	Strandebarm	Hordaland, Kvam	60°16'09.8"N, 06°00'56.8"E	hydro	10–50	*Z. noltii*
C	Gripnesvågen	Hordaland, Tysnes	60°04'00.8"N, 05°39'21.6"E	sub	50–120	*Z. noltii*
D	Huglo, Leira	Hordaland, Stord	59°51'26.9"N, 05°33'35.6"E	hydro	10–30	*Z. noltii*
E	Rødspollen	Hordaland, Sveio	59°36'07.8"N, 05°26'06.3"E	sub	70–140	*Z. marina*
F	Gjersvik	Hordaland, Etne	59°38′41.5"N, 05°55′18.8"E	sub	30–50	*R. cirrhosa*
G	Hadleholmen	Rogaland, Tysvær	59°23′44.1"N, 05°28′29.6"E	sub	30–50	*R. cirrhosa*
H	Strandnesvågen	Rogaland, Sola	58°54'26.6"N, 05°37'02.3"E	hydro	10–40	*Z. noltii*
I	Vikerøya	Vestfold, Larvik	59°02'10.9"N, 10°08'48.9"E	hydro	10–60	*Z. noltii*
J	Bliksekilen	Vestfold, Tønsberg	59°19'29.3"N, 10°29'55.5"E	hydro	10–60	*Z. noltii*
	Bliksekilen	Vestfold, Tønsberg	59°19′25.7"N, 10°29′58.2"E	hydro	10–60	*R. maritima*
K	Skjeløy	Østfold, Råde	59°17′00.4"N, 10°44′33.5"E	hydro	10–40	*R. maritima*

[1] hydro = hydrolittoral, sub = sublittoral

2.2 Extraction and purification

2.2.1 Extraction

Dried plant material was cut into small pieces before extraction with 50% aqueous methanol. The efficient ratio of the extraction solvents was determined by mixing 150 mg leaves of *Zostera marina* with 10 mL of different solvent ratios of water and methanol, from 100% water to 100% methanol. The extracts were analysed by analytical HPLC-DAD, and the peak area of rosmarinic acid and all flavonoids at 360 nm was measured. As shown in Figure H-1 (Appendix H), a solvent concentration of about 40% aqueous methanol gave the highest total peak area. The extractions were performed at room temperature for maximum 24 hours, and the extractions were repeated up to 4 times. The combined extracts were filtered through glass wool, before the methanol was removed using a rotary evaporator under reduced pressure and temperature below 30°C.

2.2.2 Liquid-liquid partitioning

The combined aqueous extracts were purified using liquid-liquid partitioning against ethyl acetate, to remove nonpolar compounds, such as chlorophylls and stilbenes from the samples.

2.2.3 Amberlite XAD-7 (adsorption chromatography)

The samples were purified by adsorption chromatography using Amberlite XAD-7 column material. Aromatic compounds, such as flavonoids, will usually be adsorbed at the column material surface, while free sugars, aliphatic acids and other non-aromatic compounds will be washed out with distilled water. After removal of unwanted compounds, the adsorbed aromatic compounds can be eluted using methanol.

2.3 Separation and isolation

2.3.1 Sixe exclusion column chromatography

Sephadex LH-20 and Toyopearl HW-40F were used as column material. Both materials separate flavonoids based on their molecule size, and flavonoids with highest molecular masses will typically elude first. As the Toyopearl HW-40F material has smaller particle sizes than Sephadex LH-20, it is considered more efficient for separation of compounds with similar size.[120]

2.3.2 Preparative High Performance Liquid Chromatography (HPLC)

The separation of flavonoids were in most cases not sufficient using the chromatographic techniques described in section 2.3.1, and preparative HPLC was applied to isolate pure compounds. The instrument used was a Gilson 321 pump equipped with an Ultimate 3000 variable wavelength detector, and a 25 × 2.2 cm (10 μm) Econosphere C18 column (Grace, Deerfield, IL). The solvents used were A) water with 0.1% formic acid and B) acetonitrile with 0.1% formic acid. Following gradients were used:

1. The initial conditions were 85% A and 15% B, followed by a linear gradient elution to 20% B (0–5 min), 30% B (5–25 min) 40% B (25–28 min), isocratic elution (28 - 30 min), and then back to 15% B.

2. Initial conditions: 90% A and 10% B, isocratic elution (0–5 min), linear gradient elution to 16% B (5–18 min), 28% B (18–22 min), 23% B (22–26 min), 28% B (26–31 min), 40% B (31–32 min), isocratic elution (32–40 min), and a final linear gradient elution back to 10% B (40–43%).

Gradient 1 was used in paper **I**, and gradient 2 was used in paper **III**. The flow rate was 15 mL/min, and aliquots of approximately 800 μL were injected.

2.4 Analytical methods

2.4.1 Analytical HPLC

Analytical HPLC is one of the most widespread method for both qualitative and quantitative analysis of flavonoids. The high pressure allows for a timesaving separation, and coupled with a UV-Vis or MS detector, the technique provides structural information about the compounds in a sample (see section 2.4.4 and 2.4.5). The instrument was an Agilent 1100 HPLC system equipped with a HP 1050 diode array detector and a 200 × 4.6 mm inside diameter, 5 μm ODS Hypersil column (Supelco, Bellefonte, PA). Two solvents, water with 0.5% TFA (A) and acetonitrile with 0.5% TFA (B) were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by a linear gradient elution to 50% B (0–30 min). The flow rate was 1.0 mL/min, and aliquots of 15 μL were injected with an Agilent 1100 series micro autosampler. The UV-Vis absorption spectra were recorded online during HPLC analysis over the wavelength range of 240–600 nm in steps of 2 nm. All samples were filtered through a 0.45 μm Millipore membrane filter before injection. In reversed-phase HPLC the stationary phase (column material) is nonpolar and the mobile phase is polar, which causes the flavonoids to separate mainly based on polarity. Glycosylation, acylation and number of hydroxyl substituent will thus influence the retention time of flavonoids, in a manner which flavonoid glycosides will elute before aglycones, and flavonoids with more hydroxyl groups will elute before less substituted analogs.[121] Sulphated flavonoids are considered more polar than the corresponding flavonoid glycosides,[122] but their estimated polarity is somewhat reduced due to interactions between the paired ions and the nonpolar reverse phase.[49] Increasing number of sugars or sulphate groups usually reduces the retention time, so the expected order of elution are diglycosides > disulphates > monoglycosides > monosulphates > aglycone, as demonstrated in Figure 1 in paper **I**. In addition acylated glycosides have longer retention times than the corresponding glycosides, as shown in Figure 1 in paper **III**.

2.4.2 Quantitative determinations

Quantitative amounts of flavonoids and polyphenolic acids in different plant material were determined by extracting four replicate plant samples with 50% aqueous methanol with magnetic stirring for 1 (paper **II**) or 2 hours (paper **II** and **III**). The number of extractions are usually of great importance for the yield. In this work samples of *Z. marina* was extracted three times (paper **II**), whereas *Z. noltii* (paper **II**) and all *Ruppia* samples (paper **III**) were extracted once. In the case of *R. cirrhosa*, the quantitative yield of chicoric acid and flavonoids after one and three extractions was determined, and as can be seen in Figure H-2 (Appendix H), the concentration of total flavonoids was somewhat higher after 3 extractions, however, the differences were not significant (p = 0.05). Accordingly, it was assumed that one extraction was sufficient to give an estimate of the quantitative content. The combined extracts were transferred into a volumetric flask to determine the total volume followed by HPLC analysis. Prior to injection, the solutions were filtered through a 0.20 (paper **II**) or 0.45 (paper **III**) µm Millipore membrane filter. The quantitative amounts of the polyphenolic compounds in paper **II** and paper **III** were determined from an HPLC calibration curve based on analytical standards, without taking into account the variation of molar absorption coefficients for individual compounds. The calibration curve was based on HPLC chromatograms recorded at 360 ± 10 nm for flavonoid standards and 330 ± 10 nm for caffeic acid standard for five (paper **II**) and six (**III**) different standard concentrations. Limit of detection (LOD) and limit of quantification (LOQ) for the reference standards were calculated based on standard deviation of y-intercepts of the regression line (SD) and the slope (S), using the equations LOD = 3.3 × SD/S and LOQ = 10 × SD/S). The accuracy of the HPLC analytical method was assessed by means of spike recovery, where known amounts of the flavonoid standards were added to the extracts (paper **II** and **III**). Percentage recovery was calculated from the equation (Eq.1):

$$\% \text{ recovery} = 100\% \times \frac{\text{Spiked sample (µg)} - \text{Unspiked sample}}{\text{Added (µg)}} \qquad \text{(Eq.1)}$$

HPLC analysis of all the samples was carried out in triplicate and the results averaged. Two sample Student's t-test assuming unequal variances with a *p*-value of 0.05 was performed.

2.4.3 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectroscopy is by far the most important method for structure elucidation of flavonoids. The assignment of proton and carbon signals in the NMR spectra is based on chemical shift values and coupling constants (*J*) from ^{1}H and ^{13}C spectra, as well as observed cross peaks in various 2D NMR experiments.[123] NMR experiments were obtained at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C on a Bruker 600 MHz instrument (paper **I**), and at 850.13 (^{1}H) and 213.77 (^{13}C) MHz on a Bruker 850 MHz instrument (paper **III**). Sample temperature was stabilized at 298 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (d_6-DMSO or d_4-MeOD) were used as secondary references (δ 39.5/2.5 and 49.1/3.31 from TMS, respectively).

1D ^{1}H NMR. The 1D proton experiment is relatively sensitive due to high abundance of the ^{1}H isotope, and provides important information about proton chemical shifts and coupling constants (J_{HH}) in a very short time. Integration of the peak areas gives quantitative information about the relative number of hydrogens in the molecule. Altogether, this information is very useful in identifying aglycone, number of sugar and acyl groups. However, for complete structural characterization, supplementary NMR methods are usually necessary.

^{13}C NMR. The 1D ^{13}C NMR experiment is less sensitive and more time consuming than the proton experiment, due to low abundance of the ^{13}C isotope (1.1%) and lower magnetogyric ratio compared to ^{1}H. The use of Distortionless Enhancement by Polarization Transfer (DEPT) experiment allows transfer from proton to carbon, thus increasing the signal strength. The experiment DEPT 135 was used to achieve accurate carbon shift values, where the signals of quaternary and CH_2 carbon are negative, and CH and CH_3 carbons are positive.

DQF-COSY. The 2D ^{1}H-^{1}H Double Quantum Filter Correlation SpectroscopY (DQF-COSY) spectra show *J*-couplings between protons, where the diagonal peaks represent

the ^{1}H spectrum, and coupling between protons are displayed as symmetrical cross peaks on each side of the diagonal.[124] This technique is frequently used in assignment of protons in glycosides.

HSQC. The 2D ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) NMR experiment shows $^{1}J_{CH}$ couplings between carbon and proton directly coupled to each other.

HSQC-TOCSY. The combined 2D method Heteronuclear Single Quantum Coherence-Total Correlation SpectroscopY (HSQC-TOCSY) shows couplings between all *J*-coupled protons in a spin system and each carbon in the same spin system. This is particularly useful when there is overlapping proton signals. Figure 11 shows the HSQC-TOCSY spectrum of a mixture of the flavonoids isorhamnetin *3-O*-β-galactopyranoside (**20**) and isorhamnetin *3-O*-β-glucopyranoside (**21**). The overlapping sugar signals can be assigned based on their J_{CH} couplings, displayed as blue (galactose or red (glucose) crosspeaks in the 2D spectrum.

HMBC. The 2D ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation NMR experiment mainly shows cross peaks between ^{2}J and ^{3}J couplings between carbon and proton. Occasionally, $^{1}J_{CH}$ and $^{4}J_{CH}$ couplings are observed. This method is particularly important for assignments of quaternary carbon, as well as determining linkage points of sugar and acyl groups.[123]

H2BC. The 2D ^{1}H-^{13}C Heteronuclear 2 Bond Correlation NMR method resembles the HMBC experiment, but differs in that only $^{2}J_{CH}$ cross peaks are observed. The method is a useful supplementary tool to HMBC, as the problem of distinguishing ^{2}J and ^{3}J bond correlations is solved.[125]

^{1}H J-resolved. The 2D homonuclear *J*-RESsolved (*J*RES) spectrum displays the chemical shift of ^{1}H along one axis, and the *J* coupling along the other axis, as shown in Figure 12. The *J*-resolved experiment helps distinguishing between different multiplets, especially when there are overlapping signals. The coupling constants for each proton signal are displayed as cross peaks along a diagonal line. Thus the *J* couplings for the H-6Aa" proton (shown as blue cross peaks) of **21** can be read directly along the vertical axis.

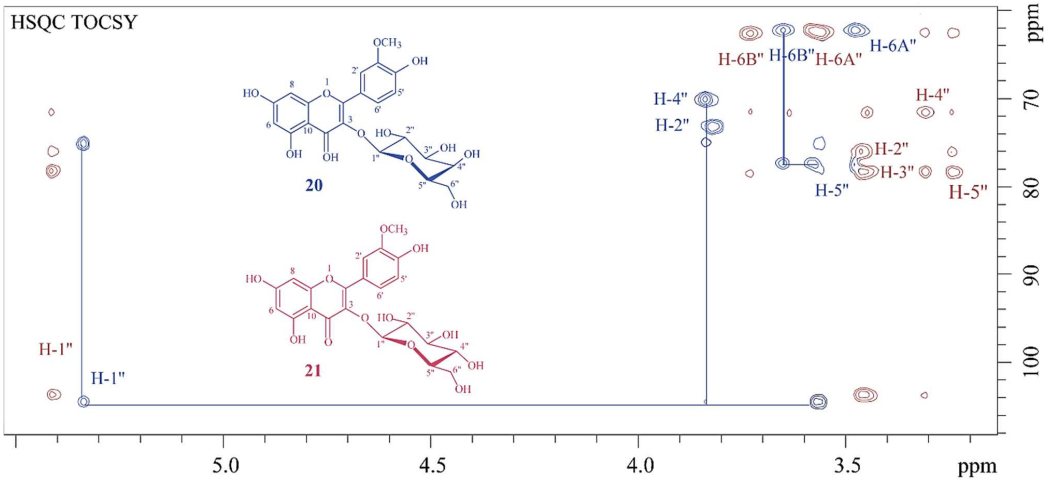


Figure 11. HSQC-TOCSY spectrum of isorhamnetin 3-*O*-β-galactopyranoside (**20**) and isorhamnetin 3-*O*-β-glucopyranoside (**21**) in d_4-MeOD recorded at 25 °C, isolated from *Ruppia cirrhosa*.

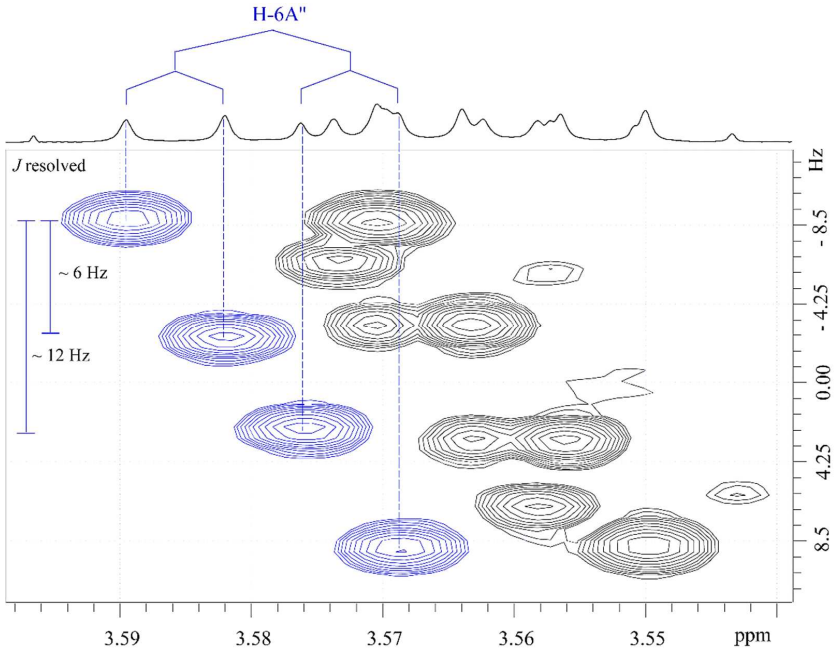


Figure 12. Example of 2D *J*-resolved spectrum of an expanded part of the sugar region of isorhamnetin 3-glucoside (**21**) in d_4-MeOD recorded at 25 °C, isolated from *Ruppia cirrhosa*. The crosspeaks for H-6A" in **21** are shown in blue.

2.4.4 Mass spectrometry (MS)

Mass spectrometry was applied to confirm structural information on flavonoids and polyphenolic compounds, as well as to determine exact masses of compounds and fragment ions. High-resolution LC-electrospray mass spectrometry (HR-LCMS) (ESI^+/TOF), spectra were recorded using a JEOL AccuTOF JMS-T100LC in combination with an Agilent Technologies 1200 Series HPLC system. A 50 × 4.6 mm internal diameter, 1.8 μm Agilent Zorbax Eclipse xDB C18 column was used for separation, and combinations of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The elution profile for HPLC consisted of initial conditions with 90% A (water with 0.1% formic acid) and 10% B (acetonitrile with 0.1% formic acid), isocratic elution 0–2 min, followed by a linear gradient elution to 50% B (2–15 min).

2.4.5 Ultraviolet/Visible Spetroscopy (UV-Vis)

UV-Visible (UV-Vis) spectra of all compounds in this work were obtained during online HPLC analysis, as described in section 2.4.1.

Flavonoids. The absorption spectra of flavonols and flavones usually consist of two bands, commonly referred to as band I (300–380 nm) and band II (240–280 nm).[126] The solvent system may influence the spectral data of compounds to some extent, but generally the UV-Vis spectral characteristics of individual flavonoids are effected by the substitution pattern on the aglycone, as well as presence of sugar and aromatic acyl groups. Electron donating substituents like methoxy- and hydroxyl groups will increase the wavelength (batochromic shift) of band I, whereas electron withdrawing substituents will lead to a hypsochromic shift of band I. Band II of flavonols and flavones usually appears as a double band, however, if the B-ring lacks substituents, or are oxygenated in the 4'-position, band II appears as a single peak,[127] as seen for apigenin in Figure 13a.

Sulphated flavonoids. Generally, flavonoid sulphates have the same UV-Vis spectral characteristics as flavonoid glycosides or their aglycones. Introducing a sulphate group on to the A-ring of a flavonoid does not influence the UV-Vis absorption significantly,

but sulphation in the 3'- or 4'-position on the B-ring will cause a large hypsochromic shift in band I, due to the electron withdrawing effect of the sulphate group,[49] as seen in Figure 3 in paper **I**.

Aromatic acids. Hydroxycinnamic acids, such as rosmarinic acid and chicoric acid, have a distinctive maximum absorption at 310–332 nm (band I), usually with a shoulder and a local UV_{max} at 227–245 nm (band II), [128] as exemplified in Figure 13b.

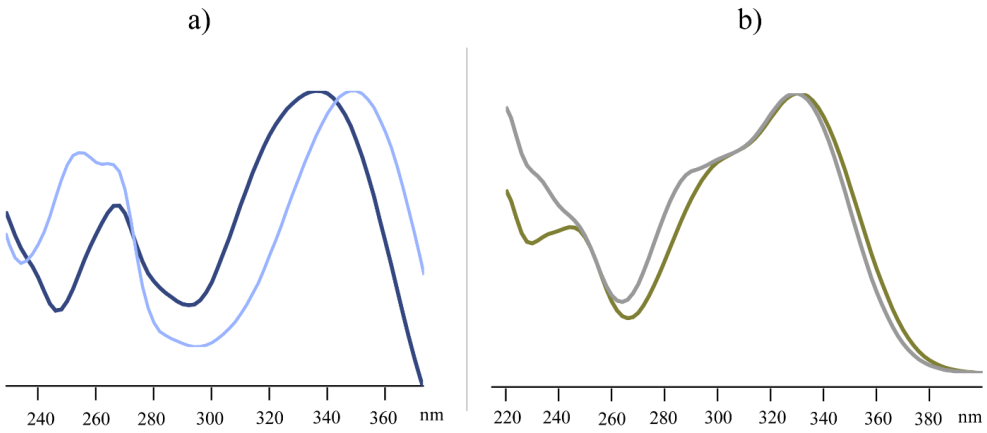


Figure 13.a) UV-Vis spectrum of luteolin (light blue) and apigenin (dark blue) recorded during online HPLC analysis of *Z. marina*; **b)** UV-Vis spectrum of chicoric acid (green) and rosmarinic acid (grey) recorded during online HPLC analysis of *Ruppia cirrhosa* and *Zostera noltii*.

2.5 Antioxidant activity

2.5.1 DPPH scavenging

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assays are widely used to determine the radical-scavenging activity of plant extracts and pure compounds. DPPH is a stable free radical having a maximum absorbance at 515–520 nm (deep purple colour). When reacting with a radical scavenger it donates a hydrogen and acquires the reduced form (pale yellow), as shown in Figure 14. The loss of purple colour correlates with the scavenging activity of the compound.

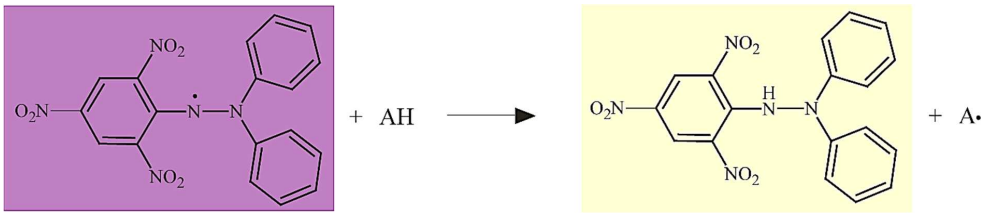


Figure 14. Reaction of DPPH radical with an antioxidant (AH)

The radical scavenging activity of a compound is commonly expressed in terms of IC_{50} values. IC_{50} denotes the concentration of sample, which is required to scavenge 50% of DPPH free radicals. The lower the IC_{50} value, the stronger the antioxidant. $IC_{50} < 50$ µg/mL indicates a very strong antioxidant, IC_{50} 50–100 µg/mL strong antioxidant, 100-150 µg/mL medium antioxidant, and $IC_{50} > 150$ µg/mL is considered weak antioxidant capacity.[129] Scavenging activity is determined by adding different concentrations of a compound to the DPPH solution. The UV-Vis absorbance at 517 nm of the DPPH solution is measured before and after addition of the sample. Figure 15 shows how the absorbance of DPPH changes with time after mixing with different concentrations of a *Ruppia cirrhosa* extract. The changes in colour from purple to yellow as the concentration of the added extract increases is also visualized.

The DPPH methods used by different research groups vary widely in regards to solvent, pH, DPPH concentration and reaction time.[130] As a result, the IC_{50} values for one compound may differ significantly depending on the method used. For instance, the IC_{50} values for the common reference compound quercetin are ranging from 0.9 to 19.3 µg/mL [87, 131-137]

The DPPH method used in this work is based on the method described by Malterud et al.[138] A Shimadzu UV-1800 UV spectrophotometer was used for the antioxidant assay. 50 µL of sample was added to 0.95 mL of a DPPH solution (45 µg/mL in methanol). The UV-Vis absorbance at 517 nm was measured every 30 seconds for 5 minutes. Percent radical-scavenging was calculated as $100 \times (A_{start} - A_{end})/(A_{start})$, where A_{start} is the absorbance before addition of the sample, and A_{end} is the absorbance value after 5 min of reaction time. Percent scavenging IC_{50} values were calculated from a

linear regression plot of percent scavenging (%) against logarithmic concentration of the test compound.

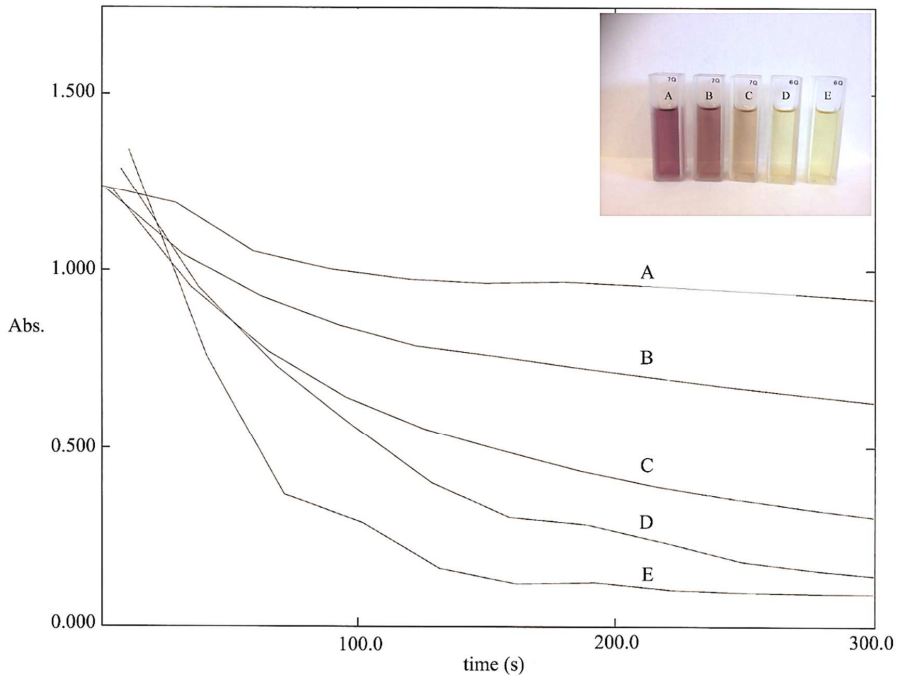


Figure 15. Absorbance vs. time (s) of DPPH solutions mixed with different concentrations (A–E) of *Ruppia cirrhosa* extract (A = 16 µg/mL, B = 32 µg/mL, C = 64 µg/mL, D = 128 µg/mL, E = 256 µg/mL).

3. RESULTS AND DISCUSSION

This chapter focuses on the main results in paper **I**–**III**. Phenolic compounds in *Zostera* and *Ruppia* plants were isolated and characterized as described in chapter 2, and the results are presented in section 3.1. Due to similarities in the flavonoid structures in this work separation of the compounds by conventional liquid chromatography (Sephadex LH-20 and Toyopearl HW-40F) was inadequate, even though several approaches with various solvent gradients of water and methanol (with and without 0.1–0.5% TFA) were tried. Most compounds were isolated with high purity by preparative HPLC as described in section 2.3.2. To achieve high resolution, the use of acidified (0.1% formic acid) solvents were necessary. Some flavonoids, in particular sulphated flavonoids, showed high instability in acidic media, and readily decomposed to their corresponding aglycones. The stability and other characteristics of sulphated flavonoids are discussed in more detail in section 3.2. Quantitative phenolic content, as well as seasonal fluctuations in *Zoster*a and *Ruppia* are addressed in sections 3.3 and 3.4. Finally, the results from DPPH scavenging assays of extracts and isolated compounds from *Ruppia cirrhosa* (paper **III**), are presented in section 3.5.

3.1 Characterization of flavonoids in marine and aquatic angiosperms

3.1.1 Characterization of flavonoids in *Zostera* (paper I and II)

Zostera marina (eelgrass) and *Zostera noltii* (dwarf-eelgrass) are marine flowering plants, mainly found in temperate regions. *Z. marina* is the most widely distributed seagrass in Norway, and is most common in southern parts of Norway north to the county border between Nordland and Troms, but has also been found further north.[139-141] *Z. noltii* is a southern, thermophilous seagrass species, distributed along the European coasts, as well as along the Northwest coast of Africa.[142-143] In Norway it is only found in the Southeast and Southwest coast.[139, 144] *Z. noltii* is a red-listed species with status as endangered (EN).

The HPLC profile of *Z. marina* extract (Figure 16a) revealed the presence of four major (**1**, **4**, **9** and **10**) and five minor flavones (**2**, **3**, **6**, **7** and **12**), together with significant amounts of rosmarinic acid (**RA**). The flavones **3**, **4**, **10**, **12** and **RA** were also found in *Z. noltii* extracts (Figure 16b), in addition to the major flavone **8**, and three minor flavones (**3**, **11** and **13**). Among the fifteen different flavones identified (Figure 17), seven were found to be sulphated (**1**, **2**, **4**, **7**–**10**).

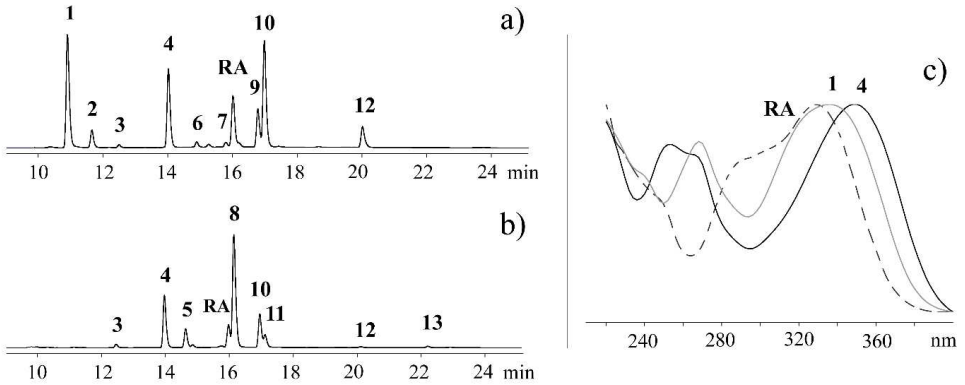
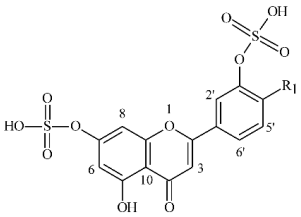
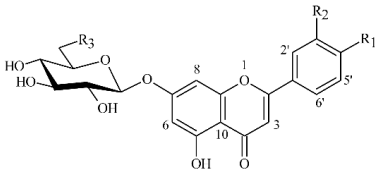


Figure 16 a-c. HPLC chromatogram of *Zostera marina* (**a**) and *Zostera noltii* (**b**) recorded at 360 ± 10 nm. **c**) UV-Vis spectrum of disulphated (**1**) and monosulphated (**4**) luteolin and rosmarinic acid (**CA**).

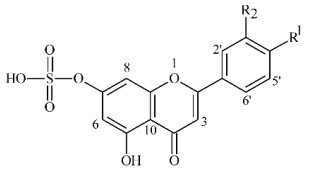
The main flavonoids in *Z. marina* were luteolin 7,3'-*O*-disulphate (**1**), luteolin 7-*O*-sulphate (**4**), chrysoeriol 7-*O*-sulphate (**9**) and diosmetin 7-*O*-sulphate (**10**), which are in accordance with previous findings.[101, 104, 108] Of these luteolin 7,3' disulphate (**1**) and chrysoeriol 7-*O*-sulphate (**9**) have not been completely assigned with NMR data before.



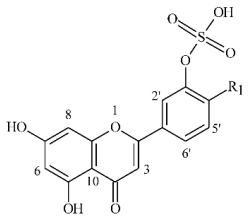
1 R_1 = OH
2 R_1 = OCH_3



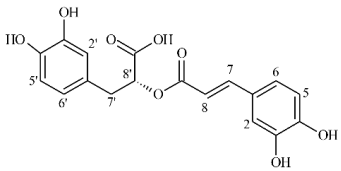
3 $R_1 = R_2 = R_3 =$ OH
5 $R_1 = R_3 =$ OH, $R_2 =$ H
6 $R_1 = R_2 =$ OH, $R_3 = O$-malonyl
11 $R_1 =$ OH, $R_2 =$ H, $R_3 = O$-malonyl
tr.2 $R_1 =$ OH or OCH_3, $R_2 =$ OCH_3 or OH, $R_3 = O$-malonyl



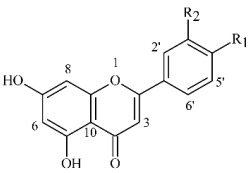
4 $R_1 = R_2 =$ OH
8 $R_1 =$ OH, $R_2 =$ H
9 $R_1 =$ OH, $R_2 =$ OCH_3
10 R_1 = OCH_3, $R_2 =$ OH



7 R_1 = OH
tr.1 R_1 = OCH_3



RA Rosmarinic acid



12 $R_1 = R_2 =$ OH
13 $R_1 =$ OH, R_2 = H
14 $R_1 =$ OH, R_2 = OCH_3
15 R_1 = OCH_3, R_2 = OH

Figure 17. Structures of the flavonoids and polyphenolic acid found in *Z. marina* and *Z. noltii* leaves. **1** = luteolin 7,3'-*O*-disulphate, **2** = diosmetin 7,3'-*O*-disulphate, **3** = luteolin 7-*O*-β-glucopyranoside, **4** = luteolin 7-*O*-sulphate, **5** = apigenin 7-glucoside, **6** = luteolin 7-*O*-β-(6"-*O*-malonyl)glucopyranoside, **7** = luteolin 3'-*O*-sulphate, **RA** = rosmarinic acid, **8** = apigenin 7-*O*-sulphate , **9** = chrysoeriol 7-*O*-sulphate, **10** = diosmetin 7-*O*-sulphate, **11**= apigenin 7-(6"-malonyl)glucoside, **12** = luteolin, **13** = apigenin, **14** = chrysoeriol, **15** = diosmetin, **tr.1** = diosmetin 3'-*O*-sulphate, **tr.2 =** chrysoeriol/diosmetin 7-(6"-malonyl)glucoside.

The main flavonoids found in *Z. noltii* were luteolin 7-*O*-sulphate (**4**), apigenin 7-*O*-sulphate (**8**) and diosmetin 7-*O*-sulphate (**10**), as well as rosmarinic acid (RA). Minor amounts of luteolin 7-*O*-β-glucoside (**3**), apigenin 7-*O*-β-glucopyranoside (**5**) and apigenin (**13**) were also identified, all of which have been found previously in *Z. noltii*.[79,

[84, 104] In addition the two Norwegian *Zostera* species contained minor amounts luteolin 7-*O*-β-(6''-*O*-malonyl)glucopyranoside (**6**) apigenin 7-(6''-malonyl)glucoside (**11**). Traces of the malonylated *O*-glycoside of chrysoeriol/diosmetin, as well as the aglycones chrysoeriol (**14**) and diosmetin (**15**) were found during HRLC-MS examinations of the extracts. The malonylated flavones **6** and **11** were identified for the first time in *Z. marina* and *Z. noltii.* Previously reported zosteric acid[79-80] was not found in any of the two examined Norwegian *Zostera* species. Other frequently occurring phenolic acids, such as caffeic, *p*-coumaric or chlorogenic acid were not detected.

*Structure elucidation of **1** and **9**.*

The ^{1}H NMR spectrum of compound **1** (Figure 18a) showed six proton signals in the aromatic region; a pair of *meta* coupled protons at δ 6.57 (1 H, *d*, J = 2.1 Hz, H-6) and δ 6.98 (1 H, *d*, J = 2.0 Hz, H-8), a one proton singlet at δ 6.74 (H-3), and the AMX system at δ 6.99 (1 H, *d*, J = 8.3 Hz, H-5'), δ 7.93 (1 H, *d*, J = 2.3 Hz, H-2'), δ 7.71 (1 H, *d*, J = 2.4, 8.7 Hz, H-6'), which were in accordance with a luteolin derivative.[145] The ^{13}C NMR values for compound **1** (Table D-1, Appendix D) were assigned on the basis of $^{1}J_{CH}$, $^{2}J_{CH}$, $^{3}J_{CH}$ and $^{4}J_{CH}$ correlations observed in the HSQC and HMBC spectra (Figures F-1 and F-2, Appendix F). The downfield carbon data for C-6, C-8 as well as the significantly downfield shifts of H-6 and H-8 strongly indicated the presence of an electron withdrawing sulphate ester in position C-7. Similarly, a second sulphate group was indicated by the NMR values of the protons H-2' and H-6', and the carbons C-2', C-4' and C-6', which were significantly shifted downfield when compared to the corresponding proton and carbon signals of luteolin. When compared to the carbon and proton values of luteolin 7,4'-*O*-disulphate,[146] the same pattern can be seem, however in compound **1**, the protons and carbons in the 2', 4' and 6'-position were shifted downfield, due to the sulphate ester group in position 3'. Compound **1** was thus identified as luteolin 7,3'-*O*-disulphate. The high resolution mass spectrum of **1** showed a positive molecular ion $[M+H]_{+}$ at m/z 446.9725 (Table 4), which confirmed the identity. The observed fragments at m/z 367.0143 and 287.0578 indicating loss of one and two

sulphate groups, were in accordance with luteolin 7-*O*-sulphate and luteolin, respectively.

The ^{1}H NMR spectrum of **9** (Figure 18b) showed signals for a pair of *meta* coupled protons at δ 6.56 (1 H, *d*, J = 2.1 Hz, H-6) and δ 7.04 (1 H, *d*, J = 2.1 Hz, H-8), a one proton doublet at δ 6.94 (1 H, *d*, J = 8.3 Hz, H-5'), a one proton doublet at 7.58 (1 H, *d*, J= 2.1 Hz, H-2′), a one proton double doublet at δ 7.60 (1 H, *dd*, J = 2.1, 8.3 Hz, H-6'), a one proton singlet at δ 6.98 (H-3), and a methoxy group at δ 3.89 (3H), corresponding to a diosmetin derivative.[79] The downfield shifts of protons H-6 and H-8 and carbon C-6 and C-8 were indicating a sulphate ester linked to the 7-position. The NMR data (Table C-1 (Appendix C) and Table D-1 (Appendix D)) were partially in accordance with previously published NMR data on diosmetin 7-*O*-sulphate,[79] but whereas the methoxy group on the B ring is in the C-4' position in diosmetin, the HMBC spectrum of compound **9** showed a long-range correlation between the methoxy protons (δ 3.89) and C-3' (δ 147.7), which verified that the methoxy group was in the C-3' position. Consequently, compound **9** was identified as chrysoeriol 7-*O*-sulphate, which was confirmed by HRLC-MS results showing a $[M+H]^+$ ion at m/z 381.0283 and a fragment at m/z 301.0719, corresponding to chrysoeriol 7-*O*-sulphate and chrysoeriol, respectively.

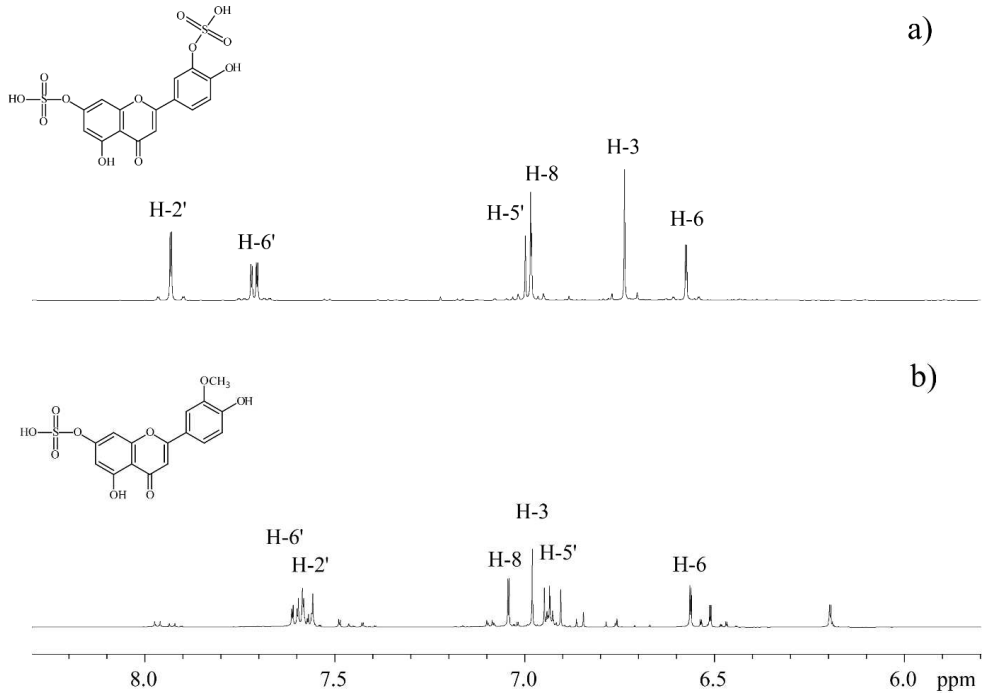


Figure 18.a) ^{1}H NMR spectrum (600.13 MHz) of luteolin 7,3'-*O*-disulphate (**1**) and **b)** chrysoeriol 7-*O*-sulphate (**9**), dissolved in d_6-DMSO, recorded at 25°C.

3.1.2 Characterization of flavonoids in *Ruppia* (paper III)

Ruppiaceae (widgeon grass) is a submersed aquatic angiosperm widely distributed in temperate and tropical regions all over the world. *Ruppia* species usually occurs in brackish or saline waters, but is also found in diluted fresh water or fresh water with high salinity, and only rarely under marine conditions.[64-66] In Norwegian coastal waters, two *Ruppia* species have been found, namely *Ruppia maritima* and *Ruppia cirrhosa.* Both species can be found in single populations with no other vascular plants present, and they are hardly ever found together. *R. maritima* can sometimes be found in proximity of *Z. noltii* populations, while *R. cirrhosa* can be found together with or close to *Z. marina* populations.

The HPLC profile of the crude extract of *R. cirrhosa* revealed one phenolic acid and eight flavonols (Figure 19a). After purification of the concentrated extract by Amberlite XAD-7 chromatography, the compounds were isolated by preparative HPLC

and analysed using high resolution LC–MS and 1D and 2D NMR spectroscopy. The compounds (Figure 20) were identified as quercetin 3-*O*-β-galactopyranoside (**16**), quercetin 3-*O*-β-glucopyranoside (**17**), quercetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside (**19**), isorhamnetin 3-*O*-β-galactopyranoside (**20**), isorhamnetin 3-*O*-β-glucopyranoside (**21**), isorhamnetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside (**22**), isorhamnetin 3-*O*-β-(6"-*O*-malonyl)glucopyranoside (**23**), and chicoric acid (**CA**) based on NMR data (Appendix C and D) and HRLC-MS values (Table 4). Quercetin 3-(6"-malonyl)glucoside (**18**) was identified by comparison with analytical standard (≥ 85% (HPLC), Sigma-Aldrich).

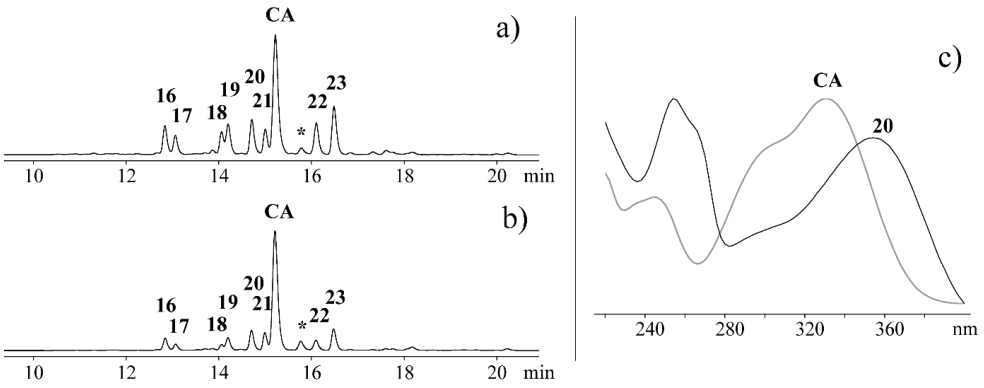
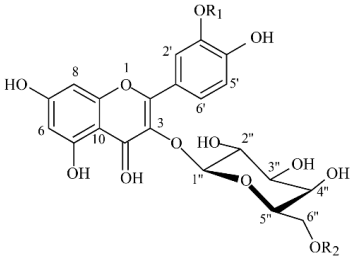
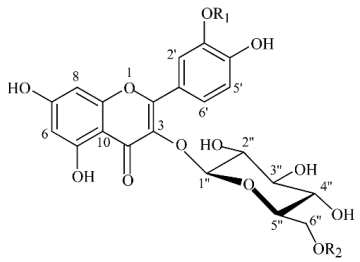


Figure 19 a-c. HPLC chromatogram of *Ruppia cirrhosa* (**a**) and *Ruppia marittima* (**b**) recorded at 360 ± 10 nm. **c**) UV-Vis spectrum of isorhamnetin 3-galactoside (**20**) and chicoric acid (**CA**). * unidentified caffeoyl

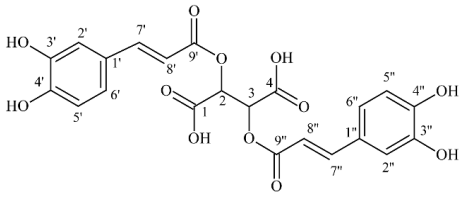
The main phenolic acid in both *Ruppia* species was chicoric acid (**CA**), which has been found previously in the seagrasses *Cymodocea nodosa* U.,[69] *Syringodium filiforme* K.,[85] *Posidionia oceania* L. [93, 117, 147] and *Thalassia hemprichii* (Ehrenb.) Ash.[109] This is the first time flavonoids (**16**–**23**) and chicoric acid have been identified in *R. cirrhosa* and *R. maritima*. The flavonols quercetin 3-*O*-glucoside and isorhamnetin 3-*O*-glucoside have previously been found in the seagrass *C. nodosa,*[69] however, this is the first report of 3-*O*-galactosides and malonylated *O*-glycosides of quercetin and isorhamnetin in aquatic plants.



16 R_1 = H, R_2 = H
19 R_1 = H, R_2 = malonyl
20 R_1= CH_3, R_2= H
22 R_1= CH_3, R_2= malonyl



17 R_1 = H, R_2 = H
18 R_1 = H, R_2 = malonyl
21 R_1= CH_3, R_2 = H
23 R_1= CH_3, R_2= malonyl



CA (chicoric acid)

Figure 20. Structures of the main phenolic compounds found in *Ruppia cirrhosa* and *Ruppia maritima*. **16** = quercetin 3-*O*-β-galactopyranoside, **17** = quercetin 3-*O*-β-glucopyranoside, **18** = quercetin 3-(6"-malonyl)glucoside, **19** = quercetin 3-*O*-β-(6"-malonyl)galactopyranoside, **20** = isorhamnetin 3-*O*-β-galactopyranoside, **21** = isorhamnetin 3-*O*-β-glucopyranoside, **CA** = chicoric acid, **22** = isorhamnetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside, **23** = isorhamnetin 3-*O*-β-(6"-*O*-malonyl)glucopyranoside.

Although the two *Ruppia* species often are found in the same habitats as *Z. marina* and *Z. noltii*, as in the case of the *R. cirrhosa* population studied in this work, *Ruppia* is considered to be more closely related to the seagrasses *P. oceania* and *C. nodosa.*[148] More recent phylogenetic studies have even assigned *Ruppia* to the Cymodoceaceae seagrass clade.[63] The phenolic similarity between the studied *Ruppia* species and previously studied *C. nodosa*[69] could therefore be seen as additional verification of the close relationship between *Ruppia* species and other seagrass members of Cymodoceaceae.

Table 4. Chromatographic and spectral (UV-Vis and MS) data of the flavonoids and phenolic acids in *Zostera* and *Ruppia*.

comp.	online HPLC			LC-MS observed		calculated mass $[M+1]^+$	molecular formula $[M+H]^+$
	t_R (min)	UV_{max}	local UV_{max}	$[M+1]^+$	fragment		
1	10.895	337	267	446.9725	367.0143, 287.0578	446.9692	$C_{15}H_{11}O_{12}S_2$
2	11.643	333	269	460.9869	381.0276, 301.0693	460.9848	$C_{16}H_{13}O_{12}S_2$
3	12.490	348	253, 266	449.1086	287.0562	449.1084	$C_{21}H_{21}O_{11}$
4	13.966	349	253, 266	367.0104	287.0557	367.0124	$C_{15}H_{11}O_9S$
5	14.629	337	266	433.1140	-	433.1135	$C_{21}H_{21}O_{10}$
6	14.848	338	252, 266	535.1080	-	535.1088	$C_{24}H_{23}O_{14}$
7	15.745	334	268	367.0127	287.0564	367.0124	$C_{15}H_{11}O_9S$
RA	15.969	330	290 (sh)	361.0929	163.0386	361.0923	$C_{18}H_{17}O_8$
8	16.137	338	267	351.0179	271.0602	351.0175	$C_{15}H_{11}O_8S$
9	16.778	348	252, 266	381.0283	301.0719	381.0280	$C_{16}H_{13}O_9S$
10	16.977	347	252, 266	381.0283	301.0719	381.0280	$C_{16}H_{13}O_9S$
11	17.114	337	267	519.1155	-	519.1139	$C_{24}H_{23}O_{13}$
12	20.010	346	250, 268	287.0553	-	287.0556	$C_{15}H_{11}O_6$
13[a]	22.203	332	268	271.0605	-	271.0607	$C_{15}H_{11}O_5$
14[a]	23.535	347	250, 268	301.0701	-	301.0712	$C_{16}H_{13}O_6$
15[a]	23.752	343	250, 268	301.0701	-	301.0712	$C_{16}H_{13}O_6$
tr.1[a]	-	-	-	381.0283	301.0719	381.0280	$C_{16}H_{13}O_9S$
tr. 2[a]	-	-	-	549.1242	-	549.1244	$C_{25}H_{25}O_{14}$
16	12.829	353	256, 264 (sh)	465.1015	303.0511	465.1029	$C_{21}H_{21}O_{12}$
17	13.055	352	256, 263 (sh)	465.0999	303.0491	465.1029	$C_{21}H_{21}O_{12}$
18	14.044	353	256, 265 (sh)	551.1060	303.0603	551.1032	$C_{24}H_{23}O_{15}$
19	14.184	354	256, 264 (sh)	551.1062	303.0603	551.1032	$C_{24}H_{23}O_{15}$
20	14.070	351	254, 266 (sh)	479.1208	317.0670	479.1184	$C_{22}H_{23}O_{12}$
21	14.990	354	254, 266 (sh)	479.1212	317.0670	479.1184	$C_{22}H_{23}O_{12}$
CA	15.206	331	245, 302 (sh)	497.0681	457. 0755, 295.0425	497.0691	$C_{22}H_{18}O_{12}Na$
*****	15.762	332	246, 302 (sh)	-	-	-	-
22	16.095	350	254, 266 (sh)	565.1216	317.1229	565.1188	$C_{25}H_{25}O_{15}$
23	16.481	355	254, 266 (sh)	565.1208	317.0691	565.1188	$C_{25}H_{25}O_{15}$

[a] only found in trace amounts in extracts; sh = shoulder; * unidentified caffeoyl

3.2 Characteristics of sulphated flavonoids (paper I)

3.2.1 UV-Vis spectroscopy

The UV-Vis absorption spectra of luteolin (**12**) and luteolin 7-*O*-sulphate (**4**) were more or less identical, and their UV_{max} values (Table 4) were consistent with previously reported data for flavones and flavone glycosides.[149] A significant hypsochromic shift in the UV_{max} of luteolin 7,3-*O*-disulphate (**1**) compared to the monosulphate (Figure 16c) was observed, indicating the presence of a sulphate group in the 3'- or 4'-position on the B ring. In addition band II appeared as a single peak instead of a double band, which is common for flavones.[127] Evidently, flavonoid sulphates seem to have analogous UV-Vis spectral characteristics as their corresponding flavonoid glycosides, however, introducing a sulphate group in 3'- or 4'-position on the B ring will cause a large hypsochromic shift in band I, and band II appears as a single peak.

3.2.2 NMR spectroscopy

Despite the lack of NMR resonances of the sulphate moieties in ^{1}H and ^{13}C NMR spectra, the linkage position of potential sulphate groups may be revealed by comparison of their spectra with spectra of their non-sulphated analogs (Table 5).

Table 5. Diagnostic ^{13}C and ^{1}H NMR sulphation shifts[1] from spectre of luteolin 7,3′-disulphate (**1a-b**), luteolin 7-O-sulphate (**4**) and chrysoeriol 7-O-sulphate (**9**)

	Position of sulphation								
	7 (**1**, **4**, **9**)		3' (**1**)			7 (**1**, **4**, **9**)		3' (**1**)	
^{13}C					^{1}H				
Ipso	-4.4 to -4.7	(C-7)	-4.5	(C-3')	Ipso	-		-	
Orto	+3.4 to +3.6	(C-6)	+7.1	(C-2')	Orto	+0.3 to +0.4	(H-6)	+0.5	(H-2')
	+3.6 to +3.9	(C-8)	+4.0	(C-4')		+0.5 to +0.6	(H-8)	-	
Meta	-1.0 to -2.2	(C-5)	-0.7	(C-1')	Meta	-		+0.1	(H-5')
	-0.8 to -1.1	(C-9)	+0.6	(C-5')		-		-	
Para	+1.9 to +2.1	(C-10)	+4.3	(C-6')	Para	-		+0.3	(H-6')

[1] δ (sulphated flavonoid) – δ (aglycone) (in ppm)

As described previously,[150] protons and carbons in *orto* and *para* positions to the sulphate ester have higher chemical shift values than their corresponding protons and

carbons of the aglycone, due to decreased shielding, whereas the carbon directly attached to the sulphate ester and the carbons in *meta* position have lower chemical shifts, due to increased shielding from the electron withdrawing sulphate ester. Both protons and carbons in positions 6, 8 and 10 on the A ring in the flavones **1**, **4** and **9** have significantly higher chemical shifts than in their corresponding aglycones, which confirms that **1**, **4** and **9** have a sulphate group connected to C-7.

The downfield shifts of C-6 and C-8 as a result of introducing a sulphate group onto the A ring is illustrated in the HSQC spectrum of luteolin 7-O-sulphate (**4**) (Figure 21). The carbon signals of **4** are displayed in red, whereas the signals of the corresponding aglycone (**12**), due to loss of sulphate group, are displayed in grey. Similar NMR shift effects caused by sulphation of the B ring of **1** were revealed. A significant increase in the shift values of C-2', C-4' and C-6', as well as a decrease in the chemical shift value of C-3' were observed, due to a sulphate ester group in the 3'-position on the B ring. The increase in the chemical shift values of H-2' and H-6' were also in accordance with a sulphate ester in the 3'-position.

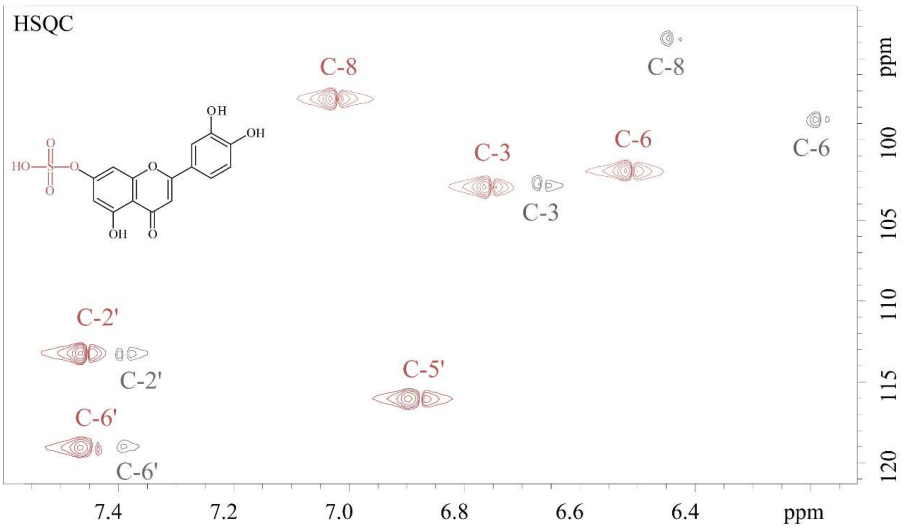


Figure 21. HSQC spectrum of compound **4** (luteolin 7-*O*-sulphate) in a mixture with **12** (luteolin). The cross peaks from compound **12** due to decomposing of **4** are shown in grey

The sulphate group induced shifts in 7-*O*-sulphate and 7,3'-O-disulphate of luteolin are illustrated in the ^{1}H NMR spectra of **1**, **4** and **12** (Figure 22). The instability of sulphated flavonoids becomes evident in this illustration, as signals corresponding to luteolin can be seen in the spectrum of luteolin 7-*O*-sulphate (**4**), due to loss of the sulphate group. It is also possible to see some weak proton signals of luteolin 7,3'-*O*-disulphate in the spectrum of luteolin (**12**), which is due to the fact that the original NMR sample contained exclusively the disulphate.

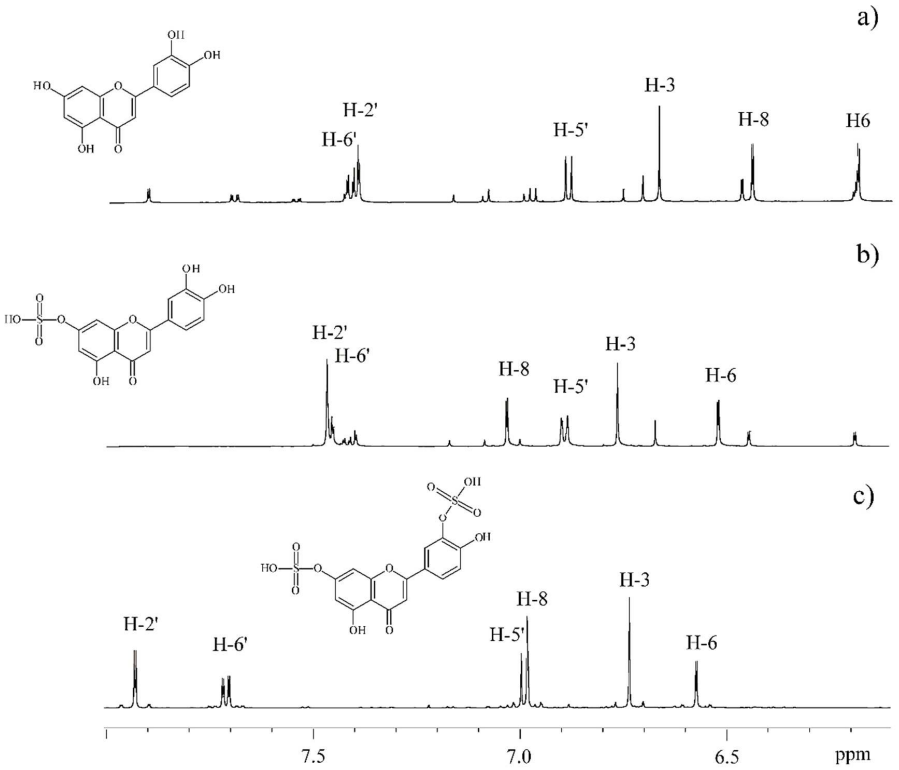


Figure 22.a) ^{1}H NMR spectra of **12** (luteolin); **b)** ^{1}H NMR spectra of **4** (luteolin 7-*O*-sulphate); **c)** ^{1}H NMR spectra of **1** (luteolin 7,3'-*O*-disulphate).

3.2.3 Stability of sulphated flavonoids in extract and as purified compounds

Stability of flavonoids in extracts. The stability of the sulphated flavones in various *Z. marina* extracts was investigated regularly during 3 months. The compounds were fairly stable in extracts containing 0.1–1.0% formic acid and in 0.1% TFA, and did not show

significant differences when compared to their storage in the corresponding neutral methanolic extract. However, in the extract containing 0.5% TFA, the flavone sulphates (**1**, **2**, **4**, **9** and **10**) decomposed gradually to their corresponding aglycones (**12**, **14** and **15**) as a result of acid hydrolysis (Figure 23). As the first replicates were analysed, the quantitative content of these sulphated flavonoids was considerably lower in the 0.5% TFA extracts than in the neutral methanolic extract, clearly indicating substantially degradation within the first few hours of extraction and analysis. The decrease of the flavonoid sulphates (**1**, **2**, **4**, **9** and **10**) was followed by a corresponding increase in the luteolin 3'-*O*-sulphate (**7**), due to loss of the sulphate group in the 7-position of (**1**). Maximum amount of the luteolin 3'-*O*-sulphate (**7**) was achieved after 1 week, before decreasing. This suggests that there has been a simultaneous generation and degradation of the 3'-*O*-sulphate, until there is no more 7,3'-*O*-disulphates left to generate 3'-*O*-sulphates, at which point the concentration of the 3'-*O*-sulphates decreases in the same manner as the 7-monosulphates.

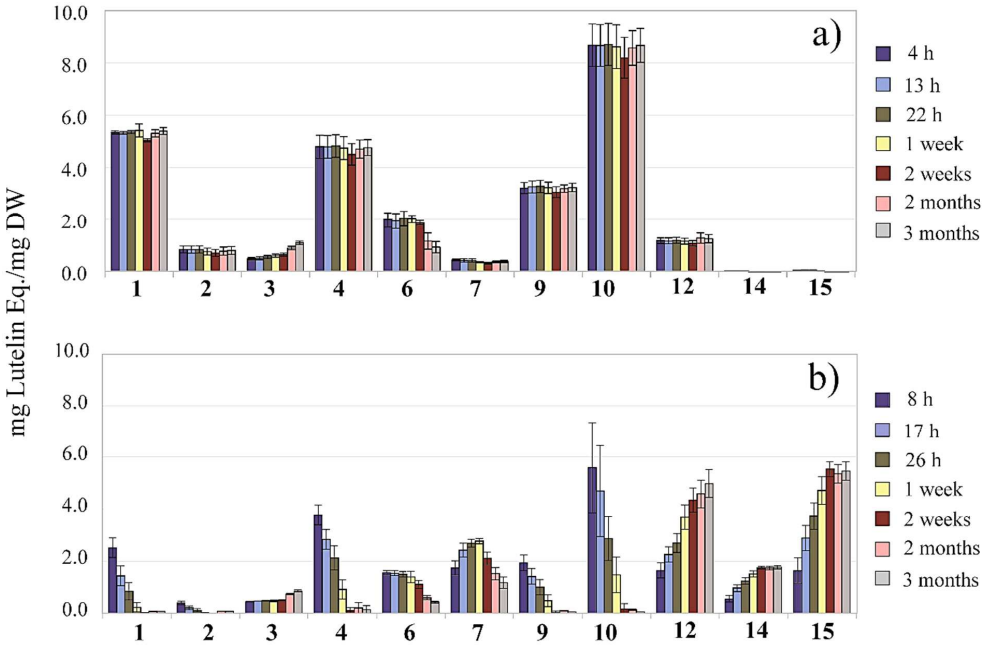


Figure 23. Flavonoid content (in mg luteolin equivalents per g DW) measured at 360 nm, hours (h), weeks and months after extraction, in 50% methanolic extracts with 0% TFA (a) and 0.5% TFA (b).

The degradation rate of disulphated flavonoids (**1** and **2**) was faster than for the monosulphates. This can partly be explained by the fact that these compounds have two sulphate groups that can be lost. Another explanation is that loss of the 3'-*O*-sulphate from the disulphate (**1**), thereby regenerating the corresponding 7-monosulphate (**4**), could to some extent counteract the degradation rate. However, the substantially increase of luteolin 3'-*O*-sulphate (**7**) during the first week suggests that loss of the sulphate group in the 7 position is more rapid and more substantial than loss of the sulphate group in the 3' position.

There was no significant change of any kind in the quantitative content of the glycosylated flavones during the first weeks, but after 2 months a decrease of luteolin 7-*O*-β-(6''-*O*-malonyl)glucopyranoside (**6**) was observed, followed by an increase of **3**, due to loss of the malonyl group. Evidently, the sulphate ester bond is less stable and more susceptible to hydrolysis under mild to moderate acidic conditions than the *O*-glycosidic linkage.

Stability of isolated flavonoid sulphates. Sulphated flavones were isolated by preparative HPLC, and their stability in the eluate solvent (consisting of water and acetonitrile with 0.1% formic acid) were monitored by analytical HPLC. The results showed that the sulphated flavones were relative stable in this solvent with a decay of 1–5% in the course of 10 days. Yet, when the solvent was removed by evaporation, these compounds quickly decomposed to their corresponding aglycones, due to accumulated acid concentrations, as exemplified in Figure 24, which shows the HPLC chromatogram of purified sample of diosmetin 7-*O*-sulphate (**10**) before evaporation and after evaporation into dryness, followed by dissolvement into d_6-DMSO. The problem with instability was partially solved by choosing different acids (formic acid or acetic acid) for the solvents, and handling the samples with great care during evaporation.

It is reasonable to assume that the occurrence of sulphated flavonoids in plants is somewhat underestimated, due to the instability of the sulphate ester bonds. Harsh extraction conditions and the often necessary use of acid during purification and

isolation may result in hydrolysis of the sulphate ester bond, thus allowing the sulphated flavonoids to escape detection.

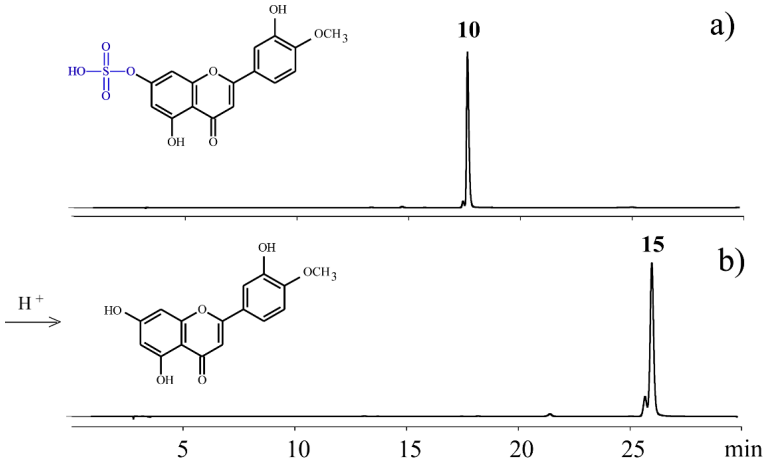


Figure 24. HPLC chromatogram of purified sample of **10** (diosmetin 7-*O*-sulphate) **a)** before evaporation; **b)** after evaporation into dryness, followed by dissolvement into d_6-DMSO.

3.3. Quantitative content of flavonoids and phenolic acids in *Zostera* and *Ruppia*

In paper **II**, the content of flavonoids and rosmarinic acid (**RA**) in *Z. marina* and *Z. noltii* from different localities (Figure 10 and Table 3 in section 2.1) was determined quantitatively. The flavonoid content was generally higher in *Z. noltii* (17.3–34.3 mg/g) than in *Z. marina* (15.0–24.5 mg/g), although regional differences within the same species were also of importance. The **RA** content was also found to be slightly higher in *Z. noltii* (2.3–4.5 mg/g) than in *Z. marina* (1.0–3.6 mg/g). In both *Ruppia* species (paper **III**) the flavonoid content was lower than in *Z. marina* and *Z. noltii*, however, the chicoric acid concentration was remarkably high, especially in *R. maritima* (27.9–30.2 mg/g).

3.3.1 Regional differences *Zostera* (paper II)

Leaves of *Z. noltii* and *Z. marina* were collected from eight localities in June 2016 and analysed for their flavonoid content. The flavonoid content was lower in *Z. marina* from Rødspollen (E) compared to the Espegrend (A) population (Table 6). A significant variation in both individual and total concentration of flavonoids in *Z. noltii* from different localities was also observed (Figure 25a). Interestingly, the flavonoid content in the two *Z. noltii* populations Huglo (D) and Gripnesvågen (C), which are in close proximity to each other, differed substantially from the other *Z. noltii* populations. Plants collected from Gripnesvågen (C), which are growing mainly sublittoral, contained the highest concentration of flavonoids (34.3 ± 1.9 mg/g) of all study sites, whilst the lowest amount of flavonoids (17.3 ± 2.9 mg/g) was observed in the hydrolittoral growing Huglo (D) population. The two *Z. noltii* populations collected on the East coast of Norway (I and J) showed similar distribution of individual flavonoids. Apigenin 7-*O*-sulphate (**8**) was the main flavonoid, followed by luteolin 7-*O*-sulphate (**4**) and diosmetin 7-*O*-sulphate (**10**). Generally, the concentrations of apigenin based flavonoids (**5**, **8** and **11**) were significantly higher in these two populations, though the relative content of sulphated flavonoids were significantly lower compared to the populations on the West coast of Norway (Figure 25b). Apigenin 7-*O*-sulphate (**8**) was also the main flavonoid in the Strandebarm population (B). However, in the other populations on the West coast, (C, D and H), the concentration of apigenin 7-*O*-sulphate (**8**) was considerably lower, and the main flavonoid was luteolin 7-*O*-sulphate (**4**), followed by diosmetin 7-*O*-sulphate (**10**).

Similar geographical differences between two *Z. noltii* populations in Cadiz and Archachon have been found.[79] In a more recent study, Grignon-Dubois and Rezzonico revealed geographical differences between *Z. noltii* samples collected at fifteen localities along the Atlantic coast and throughout the Mediterranean Sea.[81] Three different flavonoid chemotypes were identified based on their major flavonoid composition. In most populations, diosmetin 7-sulphate was the major compound (>80% of total flavonoid content), whereas in two populations apigenin 7-sulphate was main compound. Furthermore, two populations were characterized by a relative equal distribution of the 7-sulphates of diosmetin, apigenin and luteolin.

Table 6. Quantitative amounts[1] of individual flavonoids (**1**–**12**) and rosmarinic acid (**RA**) in leaves of *Z. marina* and *Z. noltii*, collected in June 2016 from eight different localities (A–J) on the west and East coast of Norway.

	1	**2**	**3**	**4**	**5**	**6**	**7**
Z. marina							
A	5.0 ± 0.4[a]	0.9 ± 0.04	0.5 ± 0.04 [b]	3.7 ± 0.7 [c]	tr	0.9 ± 0.1	0.4 ± 0.04
E	5.1 ± 0.4 [a]	1.0 ± 0.1	0.4 ± 0.04 [b]	3.2 ± 0.7 [c]	tr	0.6 ± 0.1	0.6 ± 0.04
Z. noltii							
B	nd	nd	0.9 ± 0.04[e]	7.5 ± 0.2[g,h]	1.4 ± 0.1	0.9 ± 0.1[j]	nd
C	nd	nd	1.4 ± 0.1	16.3 ± 0.8	0.9 ± 1.0	1.1 ± 0.1[j]	nd
D	nd	nd	0.9 ± 0.2	8.5 ± 1.5 [h]	0.4 ± 0.1	0.8 ± 0.2	nd
H	nd	nd	1.7 ± 0.2	9.9 ± 1.0	1.2 ± 0.1	1.5 ± 0.1[k]	nd
I	nd	nd	1.1 ± 0.1[e,f]	6.1 ± 0.7[g,i]	3.1 ± 0.3	1.2 ± 0.1[l]	nd
J	nd	nd	1.3 ± 0.1[f]	5.2 ± 0.2[i]	2.3 ± 0.1	1.4 ± 0.2[k,l]	nd

	8	**9**	**10**	**11**	**12**	**RA**	**TF**
Z. marina							
A	0.3 ± 0.01[d]	3.0 ± 0.3	7.9 ± 1.0	0.4 ± 0.1	1.6 ± 0.1	3.5 ± 0.4	24.5 ± 2.6
E	0.3 ± 0.02[d]	1.5 ± 0.2	4.6 ± 0.7	tr	0.8 ± 0.04	2.1 ± 0.1	18.1 ± 2.2
Z. noltii							
B	9.8 ± 0.1[m]	nd	4.3 ± 0.1[o,p]	1.4 ± 0.03	tr	1.3 ± 0.1	26.2 ± 0.04[x]
C	4.7 ± 0.3	nd	8.9 ± 0.7[q]	1.1 ± 0.1[s]	tr	4.5 ± 0.3	34.3 ± 1.9
D	1.4 ± 0.2	nd	4.8 ± 0.8[o]	0.5 ± 0.1	tr	3.4 ± 1.2[u]	17.3 ± 2.9
H	3.1 ± 0.3	nd	8.9 ± 0.7[q]	1.2 ± 0.1[s]	tr	2.5 ± 0.8[u,v,w]	27.5 ± 2.1 [x]
I	12.5 ± 0.7[m,n]	nd	4.0 ± 0.4 [p,r]	2.5 ± 0.3[t]	tr	2.7 ± 0.7[v]	30.5 ± 4.1[x]
J	11.4 ± 0.9[n]	nd	2.3 ± 0.3[r]	1.9 ± 0.3[t]	tr	2.1 ± 0.7[w]	27.2 ± 0.9[x]

[1] mg luteolin equivalents per g dryweight; [2] nd = not detected, tr= traces; [3] same letters (a–x) indicate where values are significantly *not* different, $p > 0.05$ with a t test; [4] Locatities: A = Espegrend, B = Strandebarm, C = Gripnesvågen, D = Huglo, E = Rødspollen, H = Strandnesvågen, I = Vikerøya, J = Bliksekilen

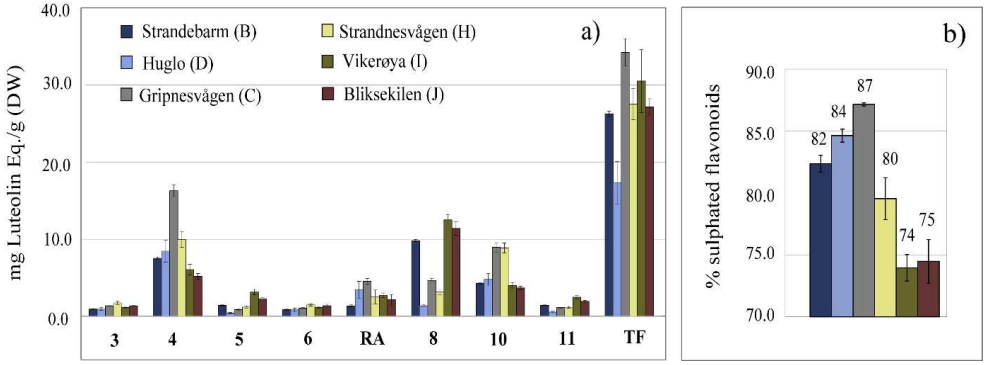


Figure 25.a) Quantitative amounts of individual flavonoids (mg Luteolin Eq./g dry weight) and rosmarinic acid (**RA**) in *Z. noltii* leaves collected in June 2016 from six different localities (C–H); **b)** % sulphated of total flavonoids in *Z. noltii* leaves from the six different localities (C–H). TF = total flavonoids.

3.3.2 Regional differences *Ruppia* (paper III)

The quantitative content of individual flavonoids (**1**–**8**) and chicoric acid was characterized in three *R. cirrhosa* and two *R. maritima* populations, collected from different localities at the East and West coast of Norway (Figure 10, Table 3 in section 2.1). The flavonoid content was significantly higher in *R. cirrhosa* from Røytepøyla (A) compared to the other *R. cirrhosa* populations on the West coast (F and G) (Table 7). No significant differences in total flavonoid or phenolic content between the two *R. maritima* populations on the East coast were observed (J and K), nonetheless some differences regarding the individual flavonoid distribution were seen. The *R. maritima* samples from Bliksekilen (J) showed a higher content of the quercetin *O*-glycosides (**16** and **17**), whereas *R. maritima* samples from the Skjeløy (K) location contained higher amounts of the malonylated isorhamnetin *O*-glycosides (**22**) and (**23**). Total flavonoid content was 5.9–14.7 mg/g (DW) for *R. cirrhosa* and 10.7 mg/g (DW) for *R. maritima*, which are somewhat lower than what was observed in *Z. marina* and *Z. noltii* in this work.

The concentrations of chicoric acid (**CA**) were significantly higher in *R. maritima* (30.2 and 27.9 mg/g) than in *R. cirrhosa* (11.1–12.7 mg/g). It seems natural to conclude

that *R. maritima* generally have a higher production of **CA**, although it should be taken into consideration that the *R. maritima* samples were collected from a different part of Norway. Differences in chicoric acid accumulation may be a function of nutritional and/or environmental stress, but there is a need for more research on how chicoric acid accumulation in plants is regulated.[151] The phenolic acid content in both *Ruppia* species was remarkably high compared to the content of rosmarinic acid found in *Z. marina* and *Z. noltii*, but was comparable to the amounts of chicoric acid found in leaves of *Cymodocea nodosa* (8.13–27.4 mg/g), *Syringodium filiforme* (0.94–5.26 mg/g) and *P. oceanica* (0.14–12.78 mg/g).[69, 85, 93, 117]

Table 7. Quantitative amounts of individual flavonoids (**16**–**23**) and chicoric acid (**CA**) in leaves of *Ruppia cirrhosa* and *Ruppia maritima* collected in summer 2017 from five localities.[1]

	16	**17**	**18**	**19**	**20**
R. cirrhosa					
A	2.2 ± 0.3 [a]	1.3 ± 0.2	0.9 ± 0.1	1.9 ± 0.3	2.9 ± 0.4
F	0.7 ± 0.1	0.5 ± 0.04[c]	0.4 ± 0.04[e]	0.7 ± 0.05[i]	1.0 ± 0.1[k]
G	1.1 ± 0.1 [b]	1.0 ± 0.1[d]	0.7 ± 0.04 [f,g]	0.8 ± 0.04[i]	1.0 ± 0.1[k]
R. maritima					
J	2.0 ± 0.5 [a]	1.0 ± 0.2 [d]	0.6 ± 0.1 [f,h]	1.5 ± 0.3[j]	1.6 ± 0.3[l]
K	1.1 ± 0.2[b]	0.6 ± 0.1[c]	0.6 ± 0.1[e,g,h]	1.4 ± 0.2[j]	2.0 ± 0.2[l]

	21	**22**	**23**	**CA**	**TF**
R. cirrhosa					
A	2.1 ± 0.2 [m]	1.1 ± 0.2[p]	2.2 ± 0.3[t]	12.7 ± 2.5[u]	14.7 ± 1.9
F	0.8 ± 0.1	0.6 ± 0.1[q,r]	1.1 ± 0.1	11.9 ± 2.2[u]	5.9 ± 0.5
G	1.3 ± 0.1 [n]	0.5 ± 0.04[q, s]	1.5 ± 0.1	11.1 ± 1.4[u]	7.9 ± 0.5
R. maritima					
J	1.7 ± 0.3[m, n, o]	0.6 ± 0.07[r, s]	1.8 ± 0.2	30.2 ± 4.3[v]	10.7 ± 1.7 [w]
K	1.6 ± 0.2[o]	1.1 ± 0.2[p]	2.3 ± 0.3 [t]	27.9 ± 5.1[v]	10.7 ± 1.5 [w]

[1]amounts are expressed in mg/g (mean value ± SD, n=4) dry weigth, based on quercetin 3-*O*-β-D-glucopyranoside (flavonoids) or caffeic acid (chicoric acid) equivalents; [2] same letters (a-w) indicate where values are significantly *not* different, $p > 0.05$ with a t test; [3] Localities: A = Røytepøyla, F = Gjersvik, G = Hadleholdmen, J = Bliksekilen, K = Skjeløy

3.4 Seasonal variation

3.4.1 Year-to-year variation in *Z. marina* (paper II)

Leaves of *Z. marina* were collected from the Espegrend locality (A) in April/May, June and in September in three subsequent years (2014–2016). Total flavonoid content for the different sampling times within the three years are shown in Figure 26a. The results show a remarkable stability from year-to-year in the flavonoid production from the spring growth in April/May to the summer flush of growth in September. The predictability was both seen for the total flavonoid production as well as for the concentrations of individual flavonoids (Figure 26b). A predictability of flavonoid production from year-to-year have previously been illustrated in leaves of the terrestrial *Artemisia tridentata subsp. wyomingensis*.[86] To our knowledge, the result presented in this work is the first report of year-to-year variation of individual flavonoid production in an aquatic plant.

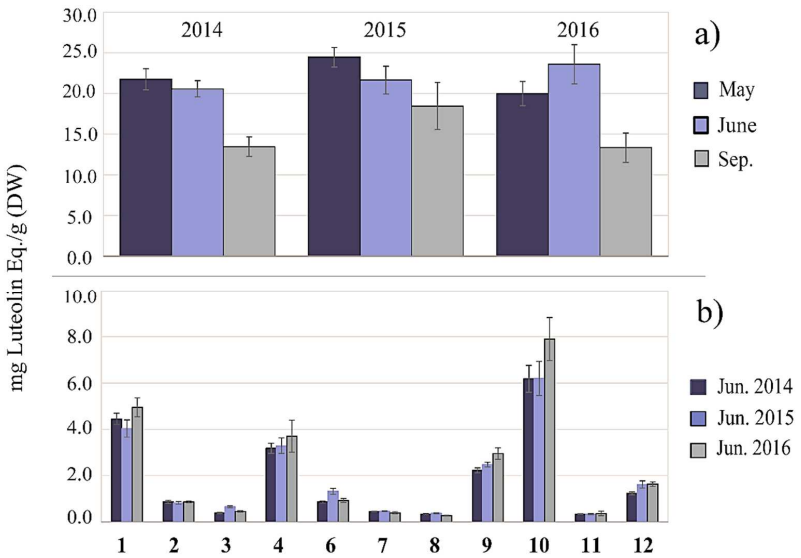


Figure 26.a) Total flavonoid content in *Z. marina* leaves collected in April/May, June and September from Espegrend (A) in 2014, 2015 and 2016; **b)** individual flavonoid content in *Z. marina* leaves collected from Espegrend (A) in June 2014–2016. The flavonoid content is shown as mg Luteolin Eq./g dry weight. Complete data in Table G-1 (Appendix G).

3.4.2. Seasonal variation in *Zostera*

Seasonal variation of flavonoids and rosmarinic acid from May 2016 to March 2017 was studied in *Z. marina* and *Z. noltii*, collected from Espegrend (A) and Huglo (D), respectively. In *Z. marina*, the flavonoid concentration (Figure 27a and Figure 28b) was generally highest in June, and lowest in September or February, though the concentration of disulphated flavonoids (**1** and **2**) appeared more or less unchanged throughout the year. In contrast, a significant increase in flavonoid content throughout the season was observed in *Z. noltii*, with peak concentration in September and February and lowest flavonoid concentration in June (Figure 27b and Figure 28b). In addition to flavonoids, rosmarinic acid (**RA**) was one of the main compounds in *Z. marina*. As in the case of flavonoids, the concentration of rosmarinic acid was higher in May and June, and considerably lower in September, March and February (Figure 28a). The rosmarinic acid content ranged from 1.0–3.6 mg/g in *Z. marina* and 2.3–3.4 mg/g in *Z. noltii* (Figure 28b). No significant seasonal variation of rosmarinic acid was observed in *Z. noltii*.

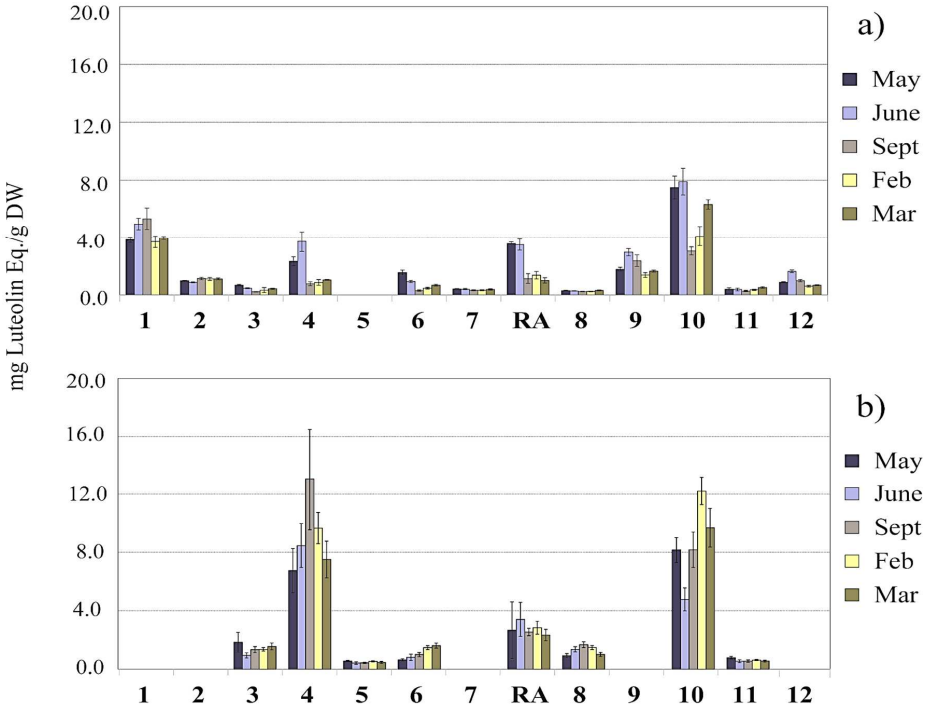


Figure 27. Quantitative amounts (mg Luteolin Eq./g dry weight) of individual flavonoids and rosmarinic acid (**RA**) in leaves of **a)** *Z. marina* from Espegrend (A) and **b)** *Z. noltii* from Huglo (D) collected in spring, summer, autumn and winter 2016–2017. Complete data in Tables G-1 and G-2 (Appendix G).

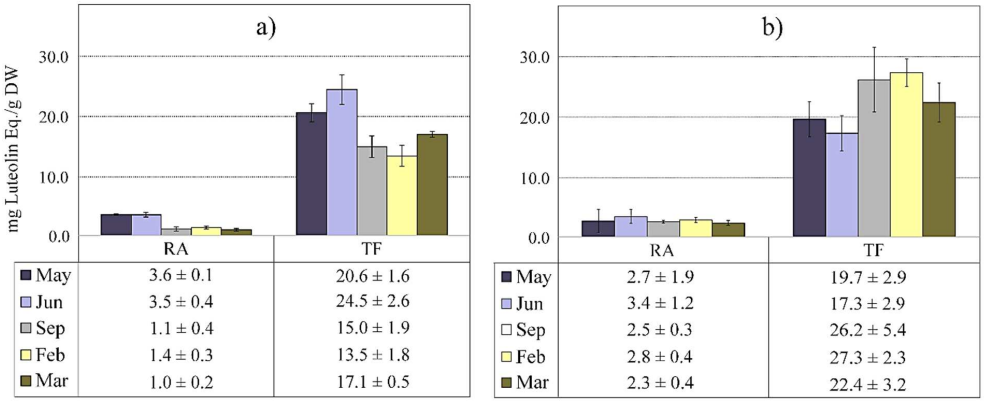


Figure 28. Quantitative amounts (mg Luteolin Eq./g dry weight) of total flavonoids (TF) and rosmarinic acid (RA) in leaves of **a)** *Z. marina* from Espegrend (A) and **b)** *Z. noltii* from Huglo (D) collected in spring, summer, autumn and winter 2016–2017.

3.4.3 Seasonal variation in *Ruppia* species (paper II)

In order to get an impression of the seasonal fluctuations of phenolics in *Ruppia*, the phenolic content of *R. cirrhosa* collected from the Røytepøyla location (A) in October, March and August was analysed. The concentration of flavonoids (Table 8 and Figure 29a) was lowest in October and March (8.4 and 11.1 mg/g, respectively) and highest in August (14.8 mg/g). Although the flavonoid concentrations differed only slightly from October to March, the most apparent difference was the distribution of individual flavonoids. In plants collected in October and August, the relative content of isorhamnetin based flavonoids (**20**–**23**) were higher compared to the quercetion based flavonoids (**16**–**19**), however, in the plants collected in March, this ratio was inversed (Figure 29b).

The variation of chicoric acid showed a different pattern, with a peak concentration in March (29.2 ± 6.3 mg/g) similar to the high concentrations of chicoric acid observed in *R. maritima* from the East coast during summertime.

Table 8. Quantitative amounts of individual flavonoids and chicoric acid in leaves of *Ruppia cirrhosa* collected from Røytepøyla (A) in october 2016, march 2017 and August 2017.[1,2]

	16	**17**	**18**	**19**	**20**
Oct 16	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1^{b}	1.1 ± 0.2	1.1 ± 0.2
Mar. 17	2.2 ± 0.4^{a}	0.8 ± 0.2	0.9 ± 0.2^{b}	1.1 ± 0.2^{c}	1.5 ± 0.3
Aug 17	2.2 ± 0.3^{a}	1.3 ± 0.2	0.9 ± 0.^{b}	1.9 ± 0.3^{c}	2.9 ± 0.4

	21	**22**	**23**	**CA**	**TF**
Oct 16	0.8 ± 0.1^{d}	1.2 ± 0.2^{e}	2.0 ± 0.3	10.6 ± 2.5^{f}	8.4 ± 1.1^{g}
Mar 17	0.7 ± 0.1^{d}	1.1 ± 0.2^{e}	1.4 ± 0.3	29.2 ± 6.3	11.1 ± 2.4^{g}
Aug 17	2.1 ± 0.2	1.1 ± 0.2^{e}	2.2 ± 0.3	12.7 ± 2.5f	14.7 ± 1.9

[1] amounts are expressed in mg/g (mean value ± SD, n=4) dry weight, based on quercetin 3-*O*-β-D-glucopyranoside (flavonoids) or caffeic acid (chicoric acid) equivalents; [2] same letters (a-g) vertically indicate where values are significantly *not* different, $p > 0.05$ with a t test

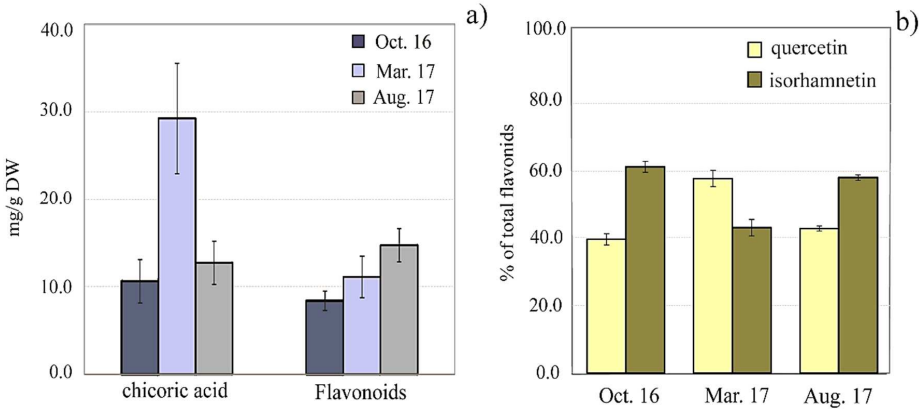


Figure 29. a) Flavonoid and chicoric acid (**CA**) content in leaves of *Ruppia cirrhosa* collected in October 2016, March 2017 and August 2017. Amounts are expressed in mg/g (mean value ± SD, n = 4) dry weight, based on quercetin 3-*O*-β-glucopyranoside (flavonoids) or caffeic acid (**CA**) equivalents; **b)** relative distribution of flavonol aglycones (% of total flavonoids) in *R. cirrhosa* collected in October, March and August (2016–2017).

3.4.4 Comparison of flavonoid content and seasonal variation in *Zostera* and *Ruppia*.

The variation of total flavonoid content in *Z. marina* (A), *Z. noltii* (D) and *R. cirrhosa* (A) from May 2016 to August 2017, is presented in Figure 30. Flavonoid content of *Z. noltii* from another locality, Strandnesvågen (H), in June 2016 and March 2017, as well as the flavonoid content of *Z. marina* from Rødspollen (E) in May, June and September 2016, have also been included. The difference between the two *Zostera* species is striking; the observed seasonal variation of flavonoids in *Z. noltii* from Huglo (D) showed an opposite pattern than what was observed in *Z. marina*. For both *Z. marina* localities, the total flavonoid content was highest in May or June, followed by a substantial drop in concentration in September. The lowest concentration in the Espegrend locality (A) was observed in February, followed by a slight increase in March. In contrast, the flavonoid content in *Z. noltii* from Huglo (D) was lowest in June and highest in February. An increased flavonoid content from June to March in the *Z. noltii* samples collected from Strandnesvågen (H), suggests a similar seasonal variation as observed in the Huglo (D) population.

The flavonoid content of *R. cirrhosa* was generally lower than in any of the examined *Z. noltii* or *Z. marina* samples, with concentrations ranging from 8.4–14.8 mg/g. Still, a similar seasonal variation as seen for *Z. marina* was observed for *R. cirrhosa*, as the flavonoid content increased from October to August.

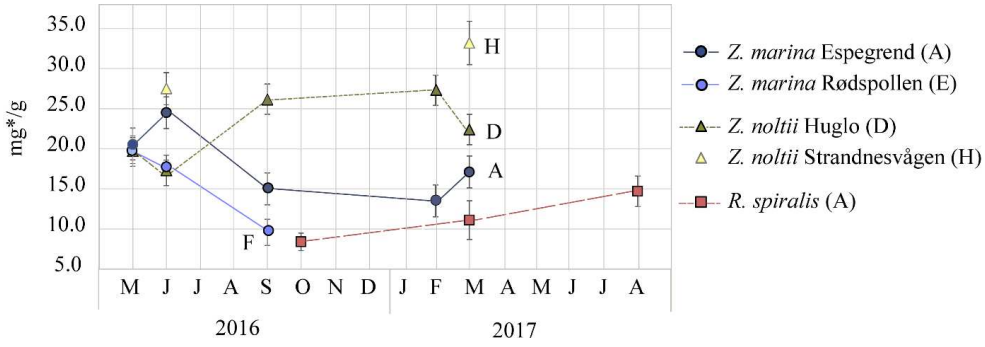


Figure 30. Observed variation of total flavonoid content in *Z. marina* from Bergen (A) and Rødspollen (E), *Z. noltii* from Huglo (D) and Strandnesvågen (C) and *R. cirrhosa* from Røytepøyla (A) in the period from May 2016 to August 2017. *Amounts are expressed in mg/g (mean value ± SD, n=4) dry weigth, based on luteolin (flavones) or quercetin 3-*O*-β-D-glucopyranoside (flavonols) equivalents

Seagrass beds of temperate and higher latitude coastal waters show considerable seasonal changes in biomass and cover.[152-153] In the subtidal areas, which are physically and biologically relatively undisturbed, *Z. marina* forms perennial populations characterized by an asexual vegetative expansion of the rhizomes.[154] To which extent *Z. marina* undergo sexual expansion in Norway is not known but low seed pollination frequency is reported at several locations.[155] During autumn and winter season, most of the leaves of *Z. marina* are reduced or wither down. At the Espegrend (A) location green leaves were partly found also in February. Similarly, leaves of *R. cirrhosa* was observed in March at location A, though during the winter months (December–February) the biomass was scarce. The thermophilous and red-listed *Z. noltii* species, only known from Southeast and Southwest parts of Norway, is perennial and mainly seen in the hydrolittoral zone.[144] *Z. noltii* also seems primarily to undergo vegetative expansion, although one can find seeds in the sediments, and flowering is observed.[144] *Z. noltii* is wintergreen and in areas where the winter is mild, the biomass is seemingly unchanged

as seen for our Huglo (D) location. This location (D) is also the densest growing *Z. noltii* population known in Norway, and interestingly this was where we measured the lowest flavonoid content of the examined *Z. noltii* species (Figure 25a). The high biomass density and lower flavonoid content may correlate to lower pressure of environmental stress factors at this site.[95, 156]

The variation of total flavonoid content of both *Z. marina* from Espegrend (A) and *Z. noltii* from Huglo (D) from May 2016 to March 2017 showed an opposite pattern (Figure 30). Interestingly, the rosmarinic acid followed the flavonoid variation seen in *Z. marina* but not to the same extent in *Z. noltii*. The seasonal variation of flavonoids in *R. cirrhosa* was comparable to that of *Z. marina*, but as opposed to the seasonal pattern of rosmarinic acid in *Z. marina*, peak concentration of chicoric acid was observed in March in *R. cirrhosa.* Ravn and co-workers reported a similar seasonal pattern for caffeic and rosmarinic acid in *Z. marina*,[83] as the one observed for the flavonoids in *Z. marina* in this study; high phenolic concentrations in spring and summer. High flavonoid concentration in spring and summer is strongly associated with environmental stress factors, mainly UV radiation – as seen for terrestrial plants.[23] It is also expected that young leaves, as they are still growing, are more vulnerable for microbial/fungal and herbivory attacks. Vergeer et al.[82] found that *Labyrinthula* infected *Z. marina* leaves indeed had a higher phenolic content than uninfected leaves. Lower temperatures correlated with higher content of phenolics, while lower than normal salinity was correlated to slightly higher phenolic content.[95] Though, the latter was not considered of great importance. The observed seasonal differences for the two *Zostera* species in this work may be related to the most obvious fact; that *Z. noltii* is a perennial, thermophilous species, increasing its flavonoid production during the colder seasons in Norway. Other factors as reproduction strategy or increased grazing pressure by swans (*Cygnus olor*) during winter season, may affect the flavonoid production as well. Opposite of the results in this work, Grignon-Dubois and Rezzonico[79] report about at decreased flavonoid production in October compared to June for *Z. noltii* samples in Spain and France. Similarly to our result, they found a higher flavonoid production in the endangered *Z. noltii* species than in *Z. marina.* The observed seasonal variation of flavonoids and phenolic acid in *R. cirrhosa* indicated a similar pattern as seen in *Z.*

marina. However, to achieve more accurate and reliable data on the seasonal variation in relation to environmental factors, a more comprehensive study of the content of both flavonoids and chicoric acid *in R. maritima* and *R. cirrhosa* is recommended.

3.5 Antioxidant properties

The antioxidant activity of *Ruppia cirrhosa* extracts and isolated compunds was assessed by DPPH radical scavenging assay in paper **III**.

3.5.1 DPPH radical scavenging capacity

The IC_{50} values of different *R. cirrhosa* extracts and isolated mixtures of flavonoids, as well as chicoric acid, are shown in Table 9. The IC_{50} values of reference compounds, 5.5 ± 0.7 µg/mL (quercetin), 11.0 ± 1.0 µg/mL (quercetin 3-*O*-β-glucoside), 13.9 ± 0.7 µg/mL (rutin), 6.1 ± 0.4 µg/mL (trolox) and 9.7 ± 1.7 µg/mL (chicoric acid), were in accordance with previous reported values.[40, 87, 118, 131, 133, 135, 137, 157-167]

Table 9. IC_{50} values of extract of *Ruppia cirrhosa* and isolated compounds from *R. cirrhosa*.

	DPPH[1] IC_{50} (µg/mL)
R. cirrhosa (Oct.)	175.7 ± 7.8
R. cirrhosa (Aug.)	152.9 ± 8.1
R. cirrhosa purified extract	31.8 ± 0.7
18 + **19**	12.1 ± 2.2
20 + **21**	88.4 ±7.0
22 + **23**	51.7 ± 6.8
chicoric acid	23.0 ± 3.2

[1] IC_{50} values calculated by linear regression of % scavenging and logarithmic concentration

The *R. cirrhosa* extract exhibited an IC_{50} value of 152.9–175.7 µg/mL, which is considered low radical scavenging activity.[168] This result is comparable to antioxidant activities of crude extracts of the seagrasses *Halodule ovalis* (IC_{50} 130 µg/mL),[169] *Syringodium isoetifolium* (IC_{50} 96.34 µg/mL), *Enhalus acoroides* (IC_{50} 115.79 µg/mL), *Cymodocea rotundata* (IC_{50} 123.72 µg/mL) and *Thalassia hemprichii* (IC_{50} 214.68 µg/mL), [170] though it must be taken into consideration that the methods used in these

studies may differ from the method used in this work. After partition with ethyl acetate, the aqueous phase of *R. cirrhosa* exhibited very strong radical scavenging activity, with an IC_{50} value of 31.8 ± 3.2 µg/mL. To our knowledge, this was the first reported results on DPPH scavenging activity of *R. cirrhosa* extracts. The extract from the plant material collected in October had a slightly lower scavenging activity than the *R. cirrhosa* extract from August. This may be related to lower phenolic content. In addition, the percent scavenging of four crude extracts of *R. cirrhosa* with known concentrations of both flavonoids and chicoric acid was examined (Figure 31), revealing a correlation between antioxidant scavenging and concentration of total flavonoids and chicoric acid.

The individual flavonoids were isolated in pairs on preparative HPLC. DPPH radical scavenging assays were performed to test the antioxidant activities of the flavonoids. The purified mixture of quercetin 3-*O*-β-(6"-*O*-malonyl)glucopyranoside (**18**) and quercetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside (**19**) showed very strong antioxidant activity, with an IC_{50} value of 12.1 ± 3.3 µg/mL, which was similar to the IC_{50} values of the reference standards quercetin (5.5 ± 0.3 µg/mL), quercetin 3-glucoside (11.0 ± 1.0 µg/mL) and rutin (13.9 ± 0.7 µg/mL) once molar mass was accounted for. The flavonoids based on the aglycone isorhamnetin showed lower antioxidant activity than that of quercetin based flavonoids, which is explained by one less free hydroxyl group on the B ring. Generally, the more hydroxyl substitutions, especially on the B ring, the stronger antioxidant activity.[171]

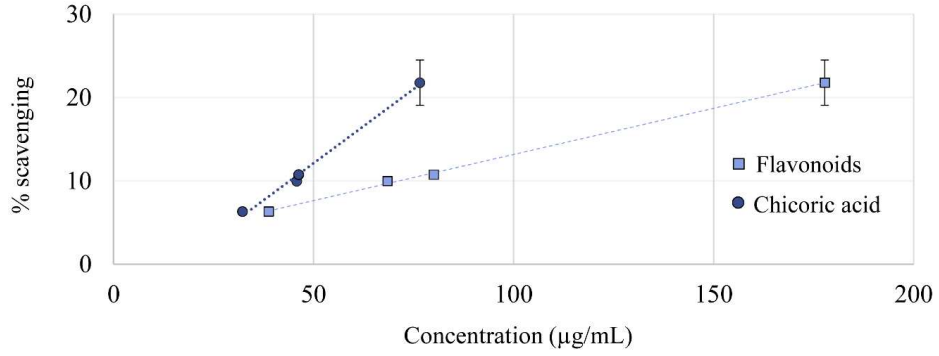


Figure 31. DPPH radical scavenging vs. concentration of chicoric acid (CA) and flavonoids (TF) in *Ruppia cirrhosa* crude extracts.

Interestingly, the malonylated isorhamnetin *O*-glycosides (**22** and **23**) showed higher antioxidant activity than the corresponding isorhamnetin *O*-glycosides (**20** and **21**), with IC_{50} values of 51.7 ± 6.8 µg/mL and 88.4 ± 7.0 µg/mL, respectively. Chicoric acid isolated from *R. cirrhosa* had a higher IC_{50} value (23.0 ± 3.2 µg/mL) than the mixture of quercetin 3-*O*-β-(6"-*O*-malonyl)glucopyranoside (**18**) and quercetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside (**19**), but had a lower IC_{50} value than the isolated isorhamnetin based flavonoids (**20** & **21** and **22** & **12**). Chicoric acid isolated in this study had a higher IC_{50} value than the reference compound. No significant impurities in the purified chicoric acid sample were observed in this study (NMR, HPLC-UV-Vis). However, water content, especially if the compound is hygroscopic, and inorganic salt content will normally not be determined by these methods.[172] Nonetheless, both isolated chicoric acid and reference compound showed very strong antioxidant activity.

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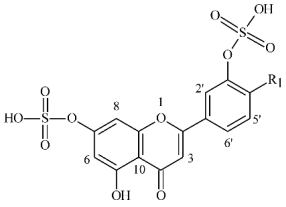
APPENDIX A–H

APPENDIX A – An overview of the compounds involved in the thesis.

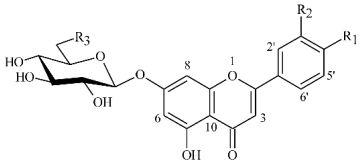
No.	Compound name	^{1}H NMR	^{13}C NMR	Paper ref.
1	Luteolin 7,3'-*O*-disulphate	Table C-1	Table D-1	I (**1**), II (**1**)
2	Diosmetin 7,3'-*O*-disulphate			I (**2**), II (**2**)
3	Luteolin 7-*O*-β-glc	Table C-1	Table D-1	I (**3**), II (**3**)
4	Luteolin 7-*O*-sulphate	Table C-1	Table D-1	I (**4**), II (**4**)
5	Apigenin 7-*O*-β-glc			II (**5**)
6	Luteolin 7-*O*-β-(6"-*O*-mal)glc	Table C-1	Table D-1	I (**5**), II (**6**)
7	Luteolin 3'-*O*-sulphate			II (**7**)
RA	Rosmarinic acid			I (**6**), II (**RA**)
8	Apigenin 7-*O*-sulphate			II (**8**)
9	Chrysoeriol 7-*O*-sulphate	Table C-1	Table D-1	I (**7**), II (**9**)
10	Diosmetin 7-*O*-sulphate			I (**8**), II (**10**)
11	Apigenin 7-*O*-β-(6"-*O*-mal)glc			II (**11**)
12	Luteolin	Table C-1	Table D-1	I (**9**), I (**12**)
13	Apigenin			II (**13**)
14	Chrysoeriol	Table C-1	Table D-1	II (**14**)
15	Diosmetin	Table C-1	Table D-1	II (**15**)
tr.1	Diosmetin 3'-*O*-sulphate			I
tr.2	Chrysoeriol/Diosmetin 7-O-β -(6"-O-mal)glc			I
16	Quercetin 3-*O*-β-gal	Table C-2	Table D-2	III (**1**)
17	Quercetin 3-*O*-β-glc	Table C-2	Table D-2	III (**2**)
18	Quercetin 3-*O*-β-(6"-*O*-mal)glc			III (**3**)
19	Quercetin 3-*O*-β-(6"-*O*-mal)gal	Table C-2	Table D-2	III (**4**)
20	Isorhamnetin 3-*O*-β-gal	Table C-3	Table D-2	III (**5**)
21	Isorhamnetin 3-*O*-β-glc	Table C-3	Table D-2	III (**6**)
CA	Chicoric acid	Table C-2	Table D-2	III (**CA**)
22	Isorhamnetin 3-*O*-β-(6"-*O*-mal)gal	Table C-3	Table D-2	III (**7**)
23	Isorhamnetin 3-*O*-β-(6"-*O*-mal)glc	Table C-3	Table D-2	III (**8**)

[1] glc = glucoside, gal = galactoside, mal = malonyl

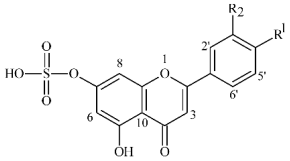
APPENDIX B – Primary structures of the compounds involved in the thesis.



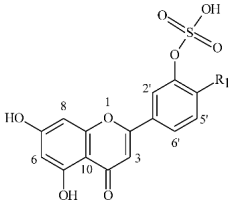
1 R_1 = OH
2 R_1 = OCH_3



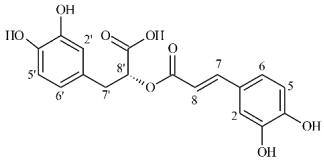
3 R_1 = R_2 = R_3 = OH
5 R_1 = R_3 = OH, R_2 = H
6 R_1 = R_2 = OH, R_3 = *O*-malonyl
11 R_1 = OH, R_2 = H, R_3 = *O*-malonyl
tr.2 R_1 = OH or OCH_3, R_2 = OCH_3 or OH, R_3 = *O*-malonyl



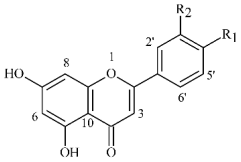
4 R_1 = R_2 = OH
8 R_1 = OH, R_2 = H
9 R_1 = OH, R_2 = OCH_3
10 R_1 = OCH_3, R_2 = OH



7 R_1 = OH
tr.1 R_1 = OCH_3

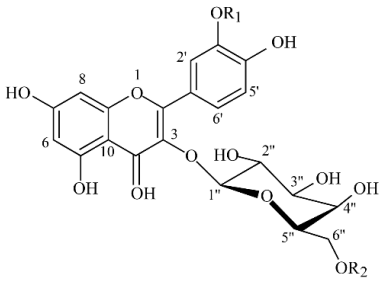


RA Rosmarinic acid

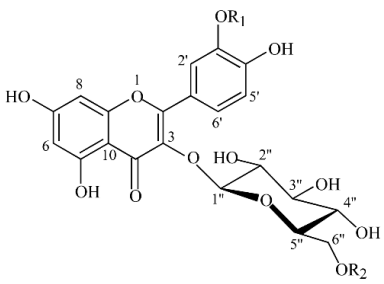


12 R_1 = R_2 = OH
13 R_1 = OH, R_2 = H
14 R_1 = OH, R_2 = OCH_3
15 R_1 = OCH_3, R_2 = OH

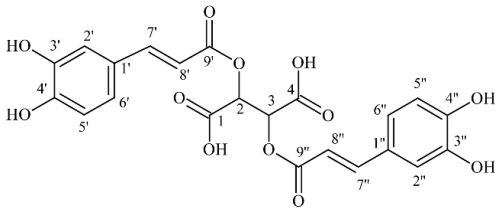
Figure B-1. Structures of phenolic compounds found in *Zostera marina* and *Z. noltii*. Structures of the flavonoids and polyphenolic acid found in *Z. marina* and *Z. noltii* leaves. **1** = luteolin 7,3'-*O*-disulphate, **2** = diosmetin 7,3'-*O*-disulphate, **3** = luteolin 7-*O*-β-glucopyranoside, **4** = luteolin 7-*O*-sulphate, **5** = apigenin 7-glucoside, **6** = luteolin 7-*O*-β-(6"-*O*-malonyl)glucopyranoside, **7** = luteolin 3'-*O*-sulphate, **RA** = rosmarinic acid, **8** = apigenin 7-*O*-sulphate, **9** = chrysoeriol 7-*O*-sulphate , **10** = diosmetin 7-*O*-sulphate, **11**= apigenin 7-(6"-malonyl)glucoside, **12** = luteolin, **13** = apigenin, **14** = chrysoeriol, **15** = diosmetin, **tr.1** = diosmetin 3'-*O*-sulphate, **tr.2 =** chrysoeriol/diosmetin 7-(6"-malonyl)glucoside.



16 R_1 = H, R_2 = H
19 R_1 = H, R_2 = malonyl
20 R_1= CH_3, R_2= H
22 R_1= CH_3, R_2= malonyl



17 R_1 = H, R_2 = H
18 R_1 = H, R_2 = malonyl
21 R_1= CH_3, R_2 = H
23 R_1= CH_3, R_2= malonyl



CA (chicoric acid)

Figure B-2. Structures of phenolic compounds found in *Ruppia cirrhosa* and *Ruppia maritima.* **16** = quercetin 3-*O*-β-galactopyranoside, **17** = quercetin 3-*O*-β-glucopyranoside, **18** = quercetin 3-(6"-malonyl)glucoside, **19** = quercetin 3-*O*-β-(6"-malonyl)galactopyranoside, **20** = isorhamnetin 3-*O*-β-galactopyranoside, **21** = isorhamnetin 3-*O*-β-glucopyranoside, **CA** = chicoric acid, **22** = isorhamnetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside, **23** = isorhamnetin 3-*O*-β-(6"-*O*-malonyl)glucopyranoside.

Table C-1. ^{1}H (600.13 MHz) NMR spectral data for flavonoids **1**, **3**, **4**, **6**, **9**, **12**, **14** and **15** isolated from *Zostera marina*. Samples of ~ 5 mg each were dissolved in d_6-DMSO at 25 °C. Sample purities: 70–95% (based on HPLC measurements (360 nm)).

	1	**3**	**4**	**6**	**9**	**12**	**14**	**15**
3	6.74 s	6.75 *s*	6.76 *s*	6.74 *s*	6.98 *s*	6.67 *s*	6.90 *s*	6.75 *s*
6	6.57 *d* 2.1	6.44 *d* 2.1	6.53 *d* 2.1	6.44 *d* 2.1	6.56 *d* 2.1	6.19 *d* 2.1	6.19 *d* 2.1	6.2 *d* 2.1
8	6.98 *d* 2.0	6.79 *d* 2.2	7.03 d 2.1	6.76 *d* 2.2	7.04 *d* 2.1	6.44 *d* 2.1	6.51 *d* 2.1	6.48 *d* 2.1
2'	7.93 *d* 2.3	7.42 *d* 2.3	7.46 d 2.1	7.42 *d* 2.2	7.58 *d* 2.1	7.46 *d* 2.1	7.56 *d* 2.1	7.43 *d* 2.3
5'	6.99 *d* 8.3	6.90 *d* 8.4	6.89 d 7.9	6.90 *d* 8.4	6.94 *d* 8.3	6.89 *d* 7.9	6.94 *d* 8.3	7.09 *d* 8.6
6'	7.71 *dd* 8.7, 2.3	7.45 *dd* 8.3, 2.3	7.45 dd 7.8, 2.4	7.45 *dd* 8.4, 2.2	7.60 *dd* 8.3, 2.2	7.45 *dd* 7.8, 2.4	7.60 *dd* 8.3, 2.1	7.54 *dd* 8.5, 2.3
OCH_3					3.89 s		3.89 *s*	3.86 *s*
1"		5.08 *d* 7.73		5.12 *d* 7.6				
2"		3.27 *m*		3.28 *m*				
3"		3.30 *m*		3.31 *m*				
4"		3.19 *m*		3.19 *m*				
5"		3.45 *m*		3.75 *m*				
6A"		3.70 *m*		4.37 *dd* 11.8, 1.9				
6B"		3.49 *m*		4.15 *dd* 11.9, 6.7				
2"'				3.37 *s*				

s, singlet, *d*, doublet; *dd*, double doublet; *t*, triplet; *m*, multiplet.

Table C-2. ^{1}H (850.13 MHz) NMR spectral data for flavonoids **16**, **17**, **19** and chicoric acid (**CA**) isolated from *Ruppia cirrhosa*. Samples of ~ 5 mg each were dissolved in d_6-DMSO at 25 °C. Sample purities: 70–95% (based on HPLC measurements (360 nm)).

	16	**17**	**19**		**CA**
6	6.20 *d* 1.9	6.20 *d* 1.9	6.20 *d* 2.0	2	5.68 *s*
8	6.41 *d* 1.9	6.41 *d* 1.9	6.40 *d* 2.0	3	5.68 *s*
2'	7.53 *d* 2.3	7.53 *d* 2.3	7.52 *d* 2.2	2′	7.10 *d* 2.1
5'	6.82 *d* 8.4	6.82 *d* 8.6	6.81 *d* 8.6	5′	6.78 *d* 8.1
6'	7.66 *dd* 8.6, 2.3	7.66 *dd* 8.6, 2.3	7.67 *dd* 8.3, 2.3	6′	7.08 *dd* 8.2, 2.1
1"	5.37 *d* 7.7	5.46 *d* 7.4	5.37 *d* 7.7	7′	7.56 *d* 15.8
2"	3.56 *m*	3.24 *t** 8.4	3.57 *m*	8′	6.36 *d* 15.8
3"	3.37 *dd* 9.6, 3.6	3.22 *t* 8.5	3.36 *dd* 8.9, 3.7	2″	7.10 *d* 2.1
4"	3.65 *m*	3.09 *d* 5.7	3.65 *m*	5″	6.78 *d* 8.1
5"	3.33 *m*	3.08 *m*	3.61 *dt* 6.2, 1.7	6″	7.08 *dd* 8.2, 2.1
6A"	3.29 *dd* 10.8. 6.0	3.32 *m* 12.0, 6.0, 2.1	4.00 *dd* 12.0, 5.8	7″	7.56 *d* 15.8
6B"	3.46 *dd* 10.8, 6.2	3.58 *m* 12.0	4.20 *dd* 12.1, 2.3	8″	6.36 *d* 15.8
2"'			3.11 *d* 16.0		

s, singlet, *d*, doublet; *dd*, double doublet; *t*, triplet; *t**(triplet like), theoretically double doublets, but appearing as triplet; *m*, multiplet.

Table C-3. ^{1}H (850.13 MHz) NMR spectral data for **20**–**23** isolated from *Ruppia cirrhosa*. Samples of ~ 5 mg each were dissolved in d_4-MeOD at 25 °C. Sample purities: 70–95% (based on HPLC measurements (360 nm)).

	20	**21**	**22**	**23**
6	6.21 *d* 1.9	6.21 *d* 1.9	6.23 *d* 2.1	6.22 *d* 2.1
8	6.41 *d* 1.9	6.41 *d* 1.9	6.44 *d* 2.1	6.44 *d* 2.1
2'	8.03 *d* 2.0	7.93 *d* 2.0	7.90 *d* 2.0	7.88 *d* 2.1
5'	6.90 *d* 8.4	6.91 *d* 8.3	6.90 *d* 8.4	6.91 *d* 8.4
6'	7.59 *d* 8.5, 2.0	7.58 *d* 8.4, 2.0	7.62 *d* 8.3, 2.1	7.61 *d* 8.5, 2.0
OCH_3	3.96 *s*	3.95 *s*	3.97 *s*	3.95 *s*
1"	5.34 *d* 7.4	5.41 *d* 7.8	5.21 *d* 7.6	5.22 *d* 7.6
2"	3.82 *dd* 9.6, 7.8	3.46 *t** 8.6	3.81 *m*	3.4 *m* 8.6
3"	3.56 *dd* 9.1, 2.8	3.45 *dd* 9.4, 8.2	3.58 *t* 9.7	3.43 *t* 8.7
4"	3.84 *dd* 3.2, 0.9	3.30 *m* 9.4	3.89 *d* 4.3	3.35 *t* 9.7
5"	3.48 *m* 8.5	3.24 *m*	3.86 *m* 9.1	3.47 *m* 8.5
6A"	3.47 *m* 11.7, 5.7, 1.7	3.57 *dd* 11.9, 5.8	4.29 *dd* 11.4, 4.4	4.19 *dd* 12.0, 5.6
6B"	3.65 *dd* 11.8, 6.1	3.73 *dd* 12.0, 2.5	4.49 *dd* 11.6, 8.4	4.33 *dd* 12.0, 2.3

s, singlet, *d*, doublet; *dd*, double doublet; *t*, triplet; *t**(triplet like), theoretically double doublets, but appearing as triplet; *m*, multiplet.

APPENDIX D – ^{13}C NMR data

Table D-1. ^{13}C (150.90 MHz) NMR spectral data for flavonoids **1**, **3**, **4**, **6**, **9**, **12**, **14** and **15** isolated from *Zostera marina.* Samples of ~ 5 mg each were dissolved in d_6-DMSO at 25 °C. Sample purities: 70–95% (based on HPLC measurements (360 nm)).

	1	**3**	**4**	**6**	**9**	**12**	**14**	**15**
2	164.0	164.5	164.2	164.3	164.0	164.2	164.0	163.5
3	103.0	103.1	102.9	103.1	103.4	102.9	103.4	103.4
4	181.9	181.8	182.1	181.8	182.0	182.1	182.0	181.7
5	160.4	161.0	160.5	161.6	160.3	160.5	161.4	161.5
6	102.2	99.4	102.3	99.3	102.3	98.7	98.9	98.9
7	159.3	162.8	156.9	162.5	159.6	164.0	163.9	164.2
8	97.7	94.6	97.8	94.6	97.9	94.1	94.0	93.8
9	156.1	156.9	156.2	156.8	156.3	157.2	157.1	157.3
10	105.4	105.1	105.4	105.3	105.7	103.5	103.6	103.7
1'	120.3	121.5	121.0	121.3	121.4	121.0	121.4	122.9
2'	120.3	113.4	113.2	113.5	110.2	113.2	110.2	112.8
3'	141.3	145.7	145.8	145.9	147.7	145.8	147.7	146.7
4'	153.7	149.8	149.7	149.6	150.6	149.7	150.6	151.1
5'	117.3	115.9	116.2	115.9	115.6	116.2	115.6	112.04
6'	123.4	119.1	119.1	119.0	120.4	119.1	120.4	118.6
OCH_3					56.0		56.0	55.7
1"		99.8		99.6				
2"		73.1		73.2				
3"		76.2		76.2				
4"		69.5		69.7				
5"		77.0		73.8				
6A"		60.5		64.1				
6B"		60.5		64.1				
1"'				166.9				
2"'				52.9				
3"'								

Table D-2. ^{13}C (213.765 MHz) NMR spectral data for the flavonols **16**, **17, 19**–**23** and chicoric acid (**CA**) isolated from *Ruppia cirrhosa*. Samples of ~ 5 mg each were dissolved in d_6-DMSO (**16**–**19,** C**A**) or d_4-MEOD (**20**–**23**) at 25 °C. Sample purities: 70–95% (based on HPLC measurements (360 nm)).

	16	**17**	**19**	**20**	**21**	**22**	**23**		**CA**
2	156.2	156.2	156.2	158.8	158.8	157.6	157.7	1	167.6
3	133.5	133.3	133.4	135.6	135.5	134.0	135.6	2	70.7
4	177.5	177.4	177.1	179.6	179.6	178.0	173.9	3	70.7
5	161.2	161.2	161.2	163.3	163.3	161.7	161.7	4	167.6
6	98.7	98.7	98.6	100.0	100.0	98.5	98.5	1′	125.2
7	164.2	164.2	164.1	166.1	166.1	164.6	164.6	2′	115.3
8	93.5	93.5	93.4	94.9	94.9	93.6	93.6	3′	145.6
9	156.3	156.3	156.3	158.6	158.6	157.0	157.1	4′	148.9
10	103.9	104.0	103.8	105.9	105.9	104.3	104.3	5′	115.8
1'	121.1	121.2	121.5	123.2	123.2	121.5	121.6	6′	121.7
2'	116.0	116.2	116.2	114.7	114.6	113.0	113	7′	147.0
3'	144.9	144.8	144.7	148.6	148.6	146.9	147	8′	112.3
4'	148.5	148.5	148.4	151.0	151.0	149.5	149.6	9′	165.5
5'	115.2	115.2	115.1	116.1	116.1	114.7	114.6	1″	125.2
6'	122.0	122.0	121.9	123.8	122.5	122.4	122.7	2″	115.3
OCH_3				57.0	57.0	55.4	55.4	3″	145.6
1"	101.8	100.8	101.7	104.5	103.7	103.3	103.1	4″	148.9
2"	71.2	74.1	71.0	73.3	76.1	71.4	74.2	5″	115.8
3"	73.2	76.5	73.1	75.2	78.2	73.4	76.5	6″	121.7
4"	67.9	69.9	67.9	70.2	71.6	69.0	69.9	7″	147.0
5"	75.9	77.6	72.4	77.4	78.7	73.4	74.4	8″	112.3
6A"	60.1	60.9	63.5	62.3	62.7	63.1	63.4	9″	165.5
6B"	60.1	60.9	63.5	62.3	62.7	63.1	63.4		
1"'			166.5			166.3	169.0		
2"'			41.0						
3"'			167.7						

APPENDIX E – ^{1}H NMR spectra

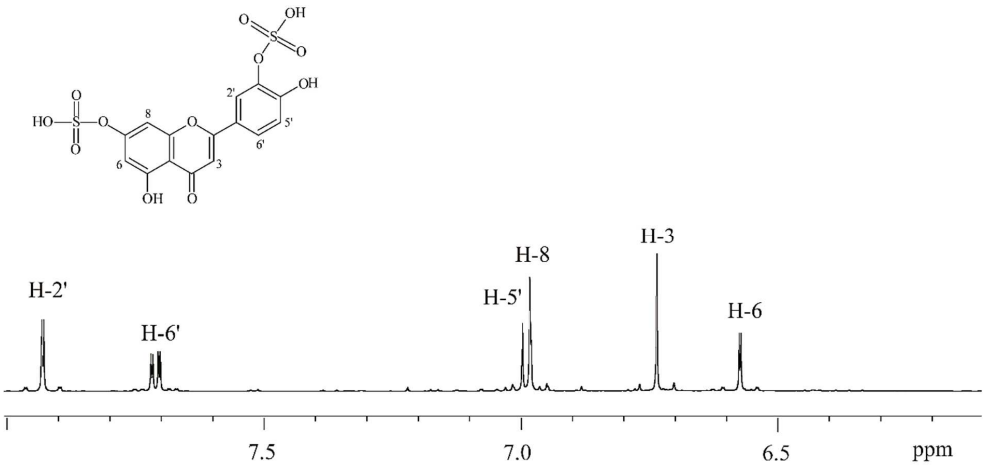


Figure E-1. ^{1}H NMR spectrum (600.13 MHz) of luteolin 7,3'-*O*-disulphate (**1**), dissolved in d_6-DMSO, recorded at 25°C.

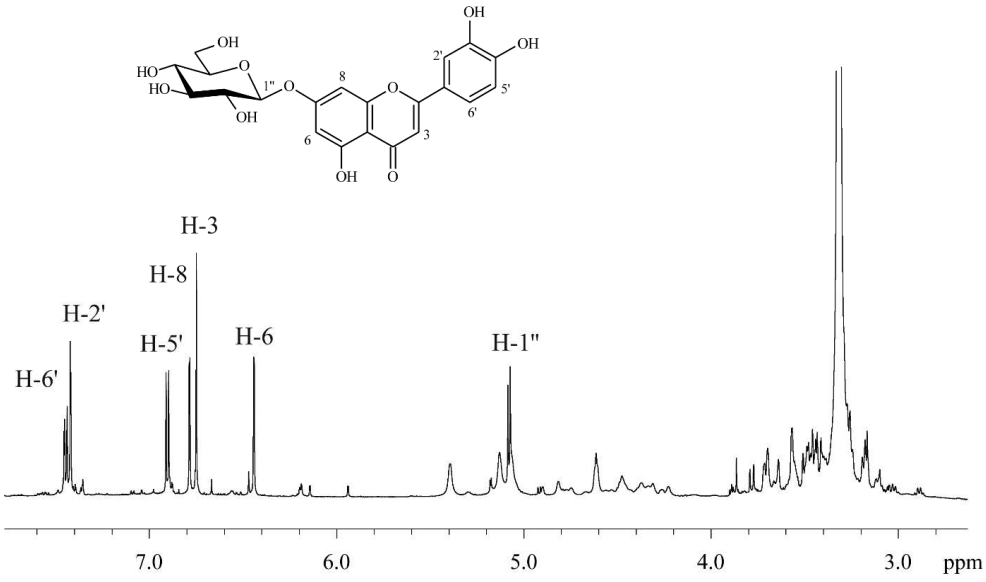


Figure E-2. ^{1}H NMR spectrum (600.13 MHz) of luteolin 7-*O*-β-glucoside (**3**), dissolved in d_6-DMSO, recorded at 25°C.

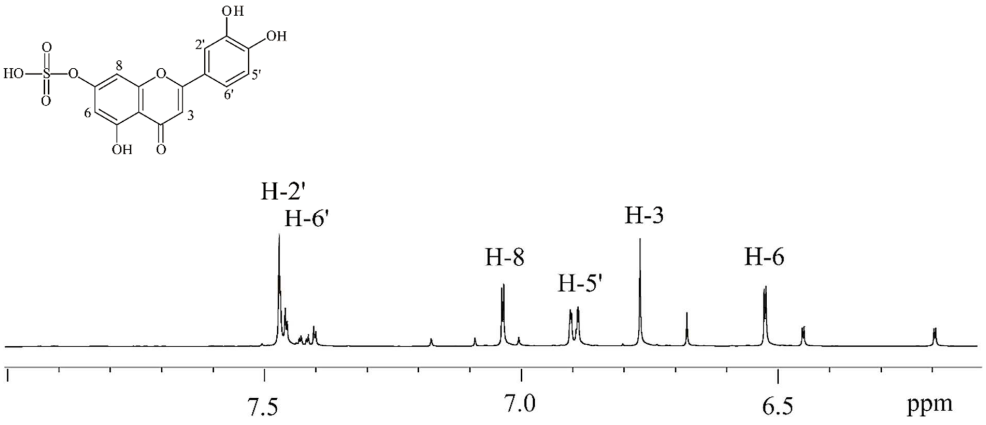


Figure E-3. ^{1}H NMR spectrum (600.13 MHz) of luteolin 7-*O*-sulphate (**4**), dissolved in d_6-DMSO, recorded at 25°C.

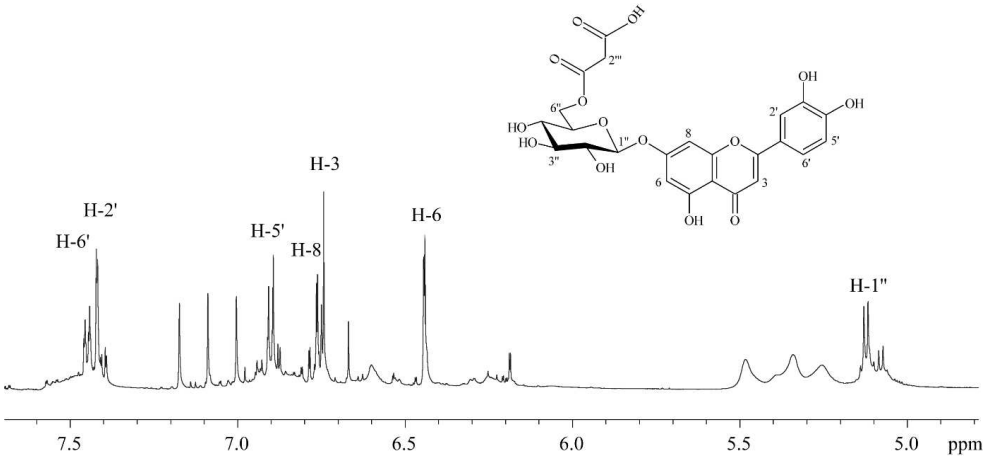


Figure E-4. ^{1}H NMR spectrum (600.13 MHz) of luteolin 7-*O*-β-(6''-*O*-malonyl)glucoside (**6**), dissolved in d_6-DMSO, recorded at 25°C.

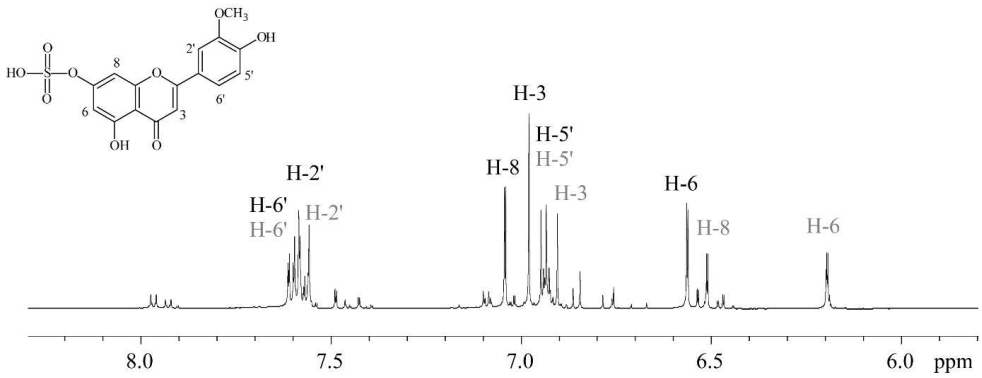


Figure E-5. ^{1}H NMR spectrum (600.13 MHz) of chrysoeriol 7-*O*-sulphate (**9**) and chrysoeriol (grey), dissolved in d_6-DMSO, recorded at 25°C.

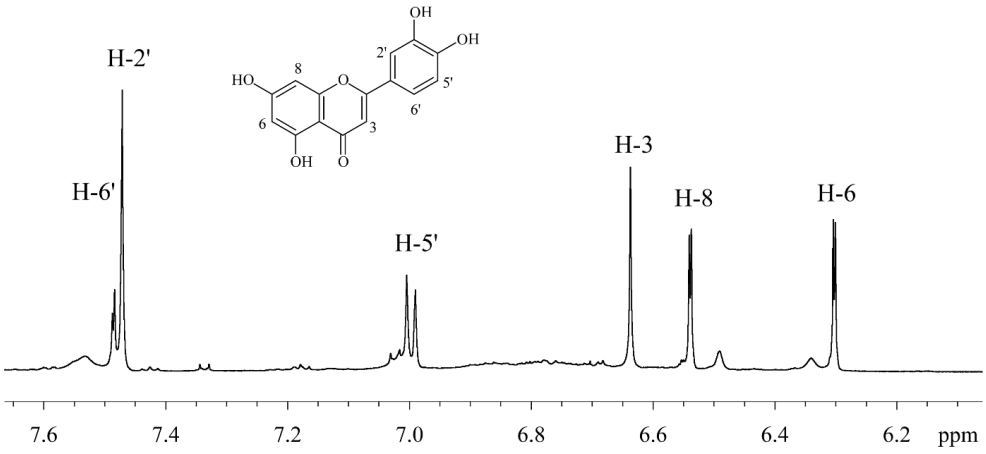


Figure E-6. ^{1}H NMR spectrum (600.13 MHz) of luteolin (**12**), dissolved in d_6-DMSO, recorded at 25°C.

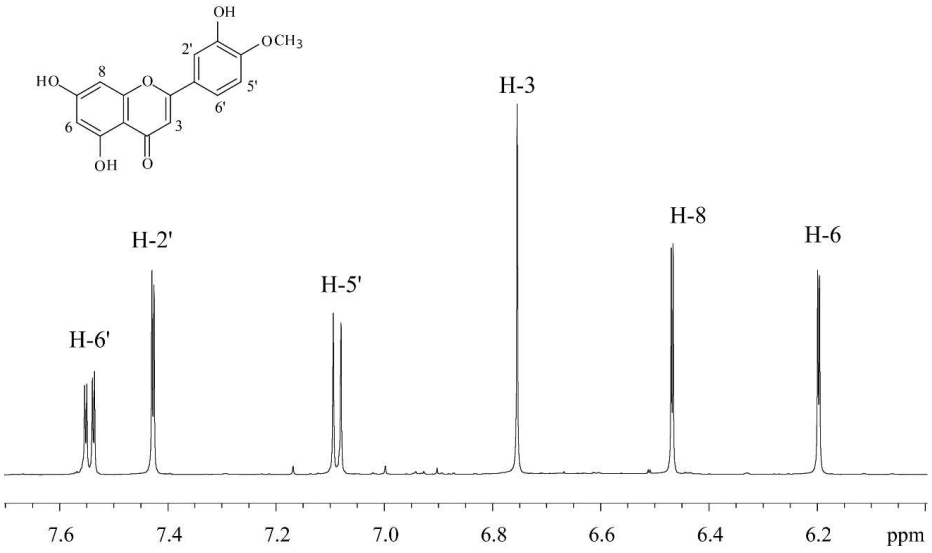


Figure E-7. [1]H NMR spectrum (600.13 MHz) of diosmetin (**15**), dissolved in d_6-DMSO, recorded at 25°C.

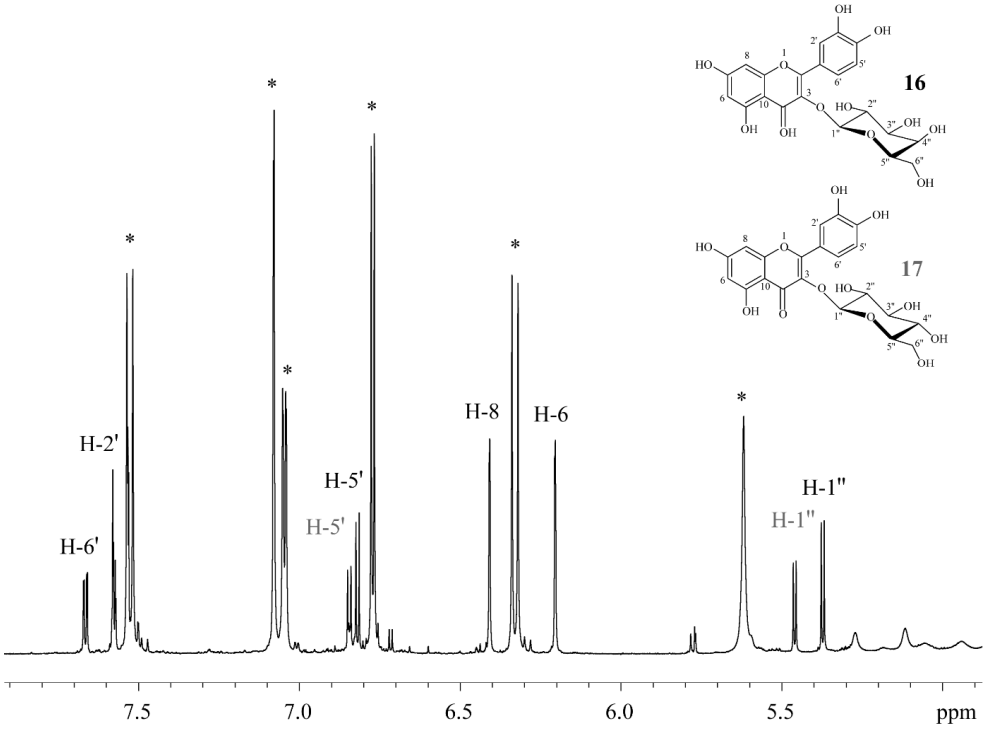


Figure E-8. [1]H NMR spectrum (850.13 MHz) of quercetin 3-*O*-galactoside (**16**) and quercetin 3-*O*-glucoside (**17**), dissolved in d_6-DMSO, recorded at 25°C. * signals from chicoric acid

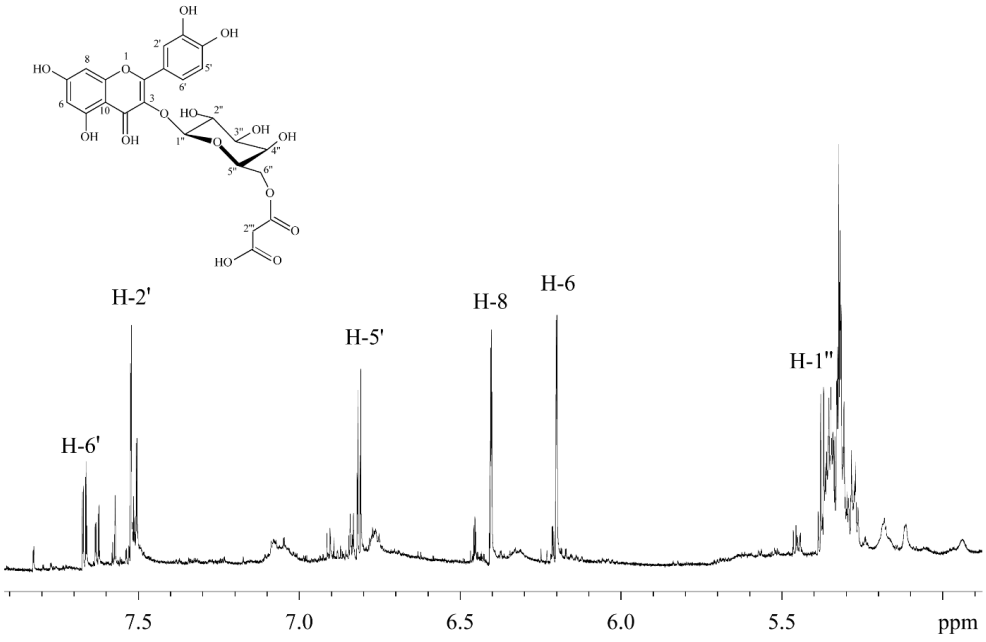


Figure E-9. ^{1}H NMR spectrum (850.13 MHz) of quercetin 3-*O*-(6''-*O*-malonyl)galactoside (**19**), dissolved in d_6-DMSO, recorded at 25°C.

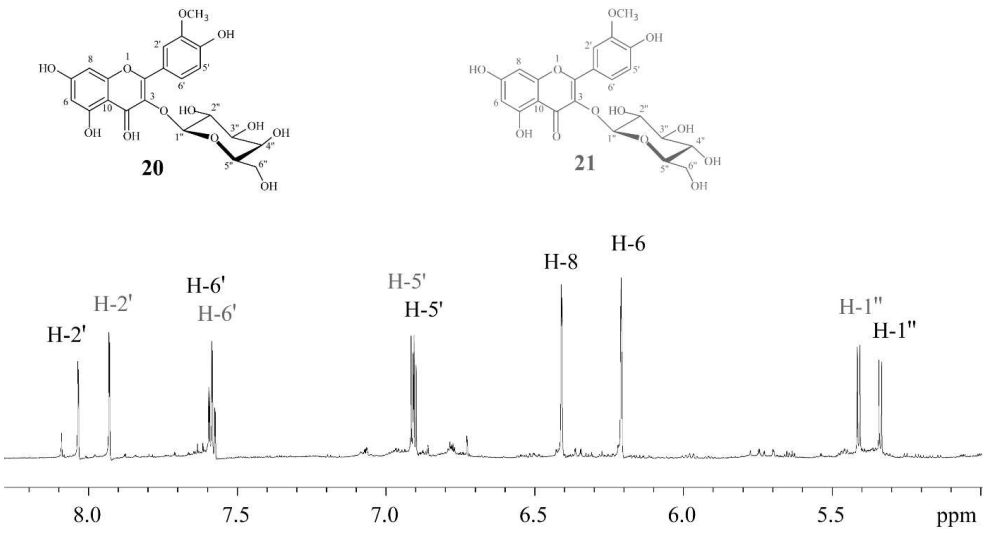


Figure E-10. ^{1}H NMR spectrum (850.13 MHz) of isorhamnetin 3-*O*-galactoside (**20**) and isorhamnetin 3-*O*-glucoside (**21**), dissolved in d_4-MeOD, recorded at 25°C.

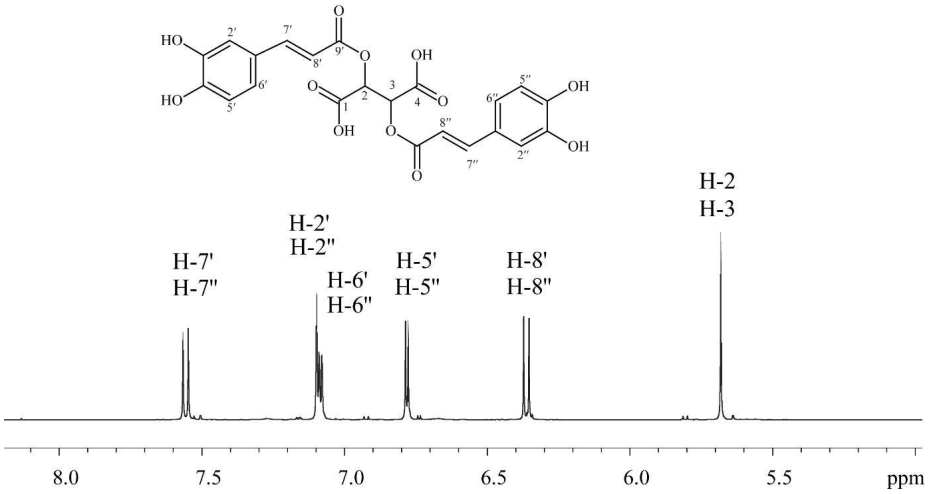


Figure E-11. ^{1}H NMR spectrum (850.13 MHz) of chicoric acid, dissolved in d_6-DMSO, recorded at 25°C.

APPENDIX F – ^{13}C and 2D NMR spectra

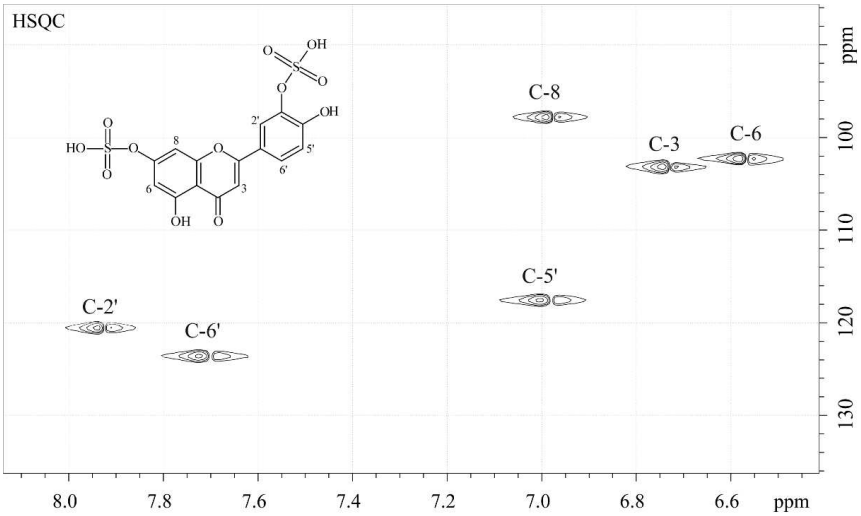


Figure F-1. HSQC spectrum of luteolin 7,3'-*O*-disulphate (**1**), dissolved in d_6-DMSO, recorded at 25°C.

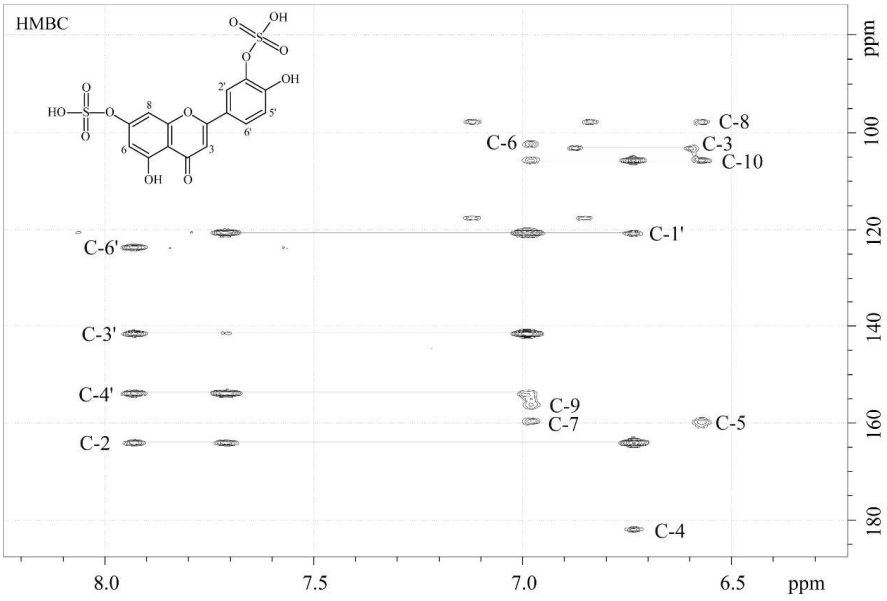


Figure F-2. HMBC spectrum of luteolin 7,3'-*O*-disulphate (**1**), dissolved in d_6-DMSO, recorded at 25°C.

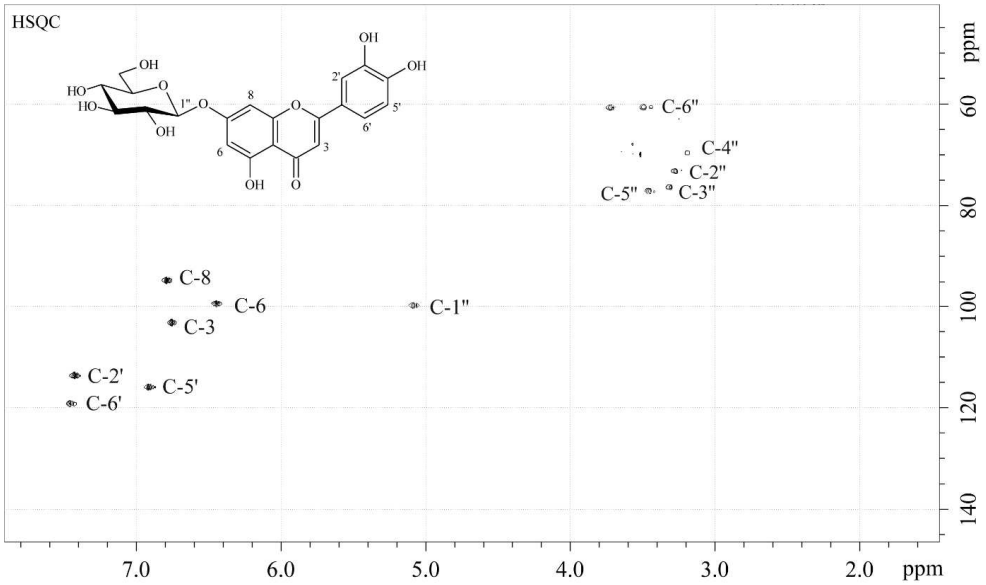


Figure F-3. HSQC spectrum of luteolin 7-*O*-β-glucoside (**3**), dissolved in d_6-DMSO, recorded at 25°C.

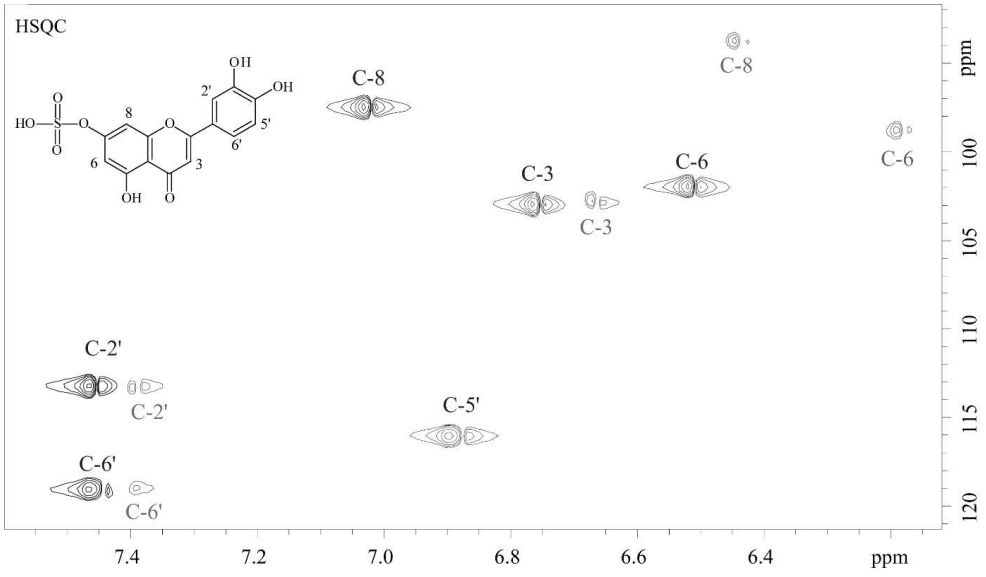


Figure F-4. HSQC spectrum of luteolin 7-*O*-sulphate (**4**), dissolved in d_6-DMSO, recorded at 25°C.

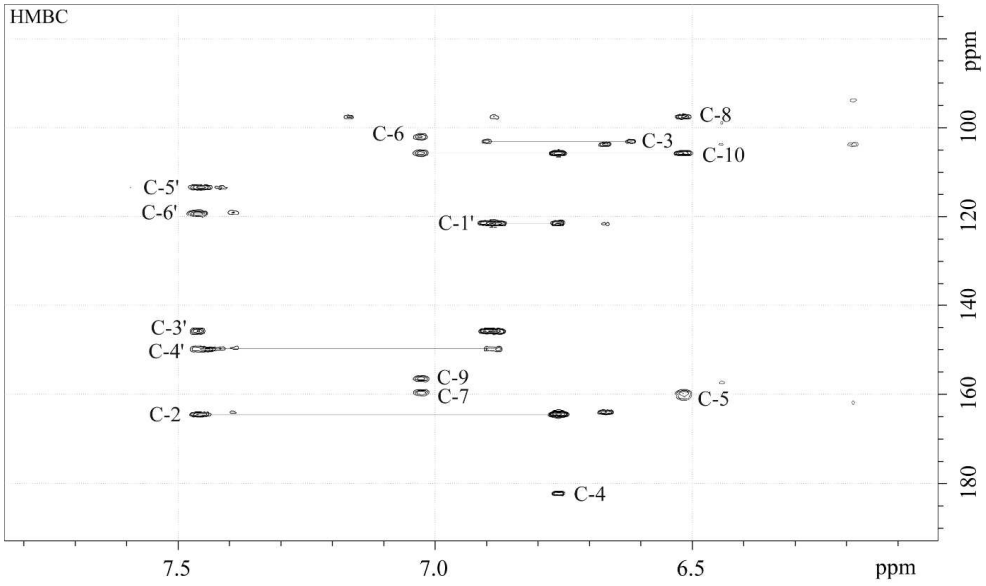


Figure F-5. HMBC spectrum of luteolin 7-*O*-sulphate (**4**), dissolved in d_6-DMSO, recorded at 25°C.

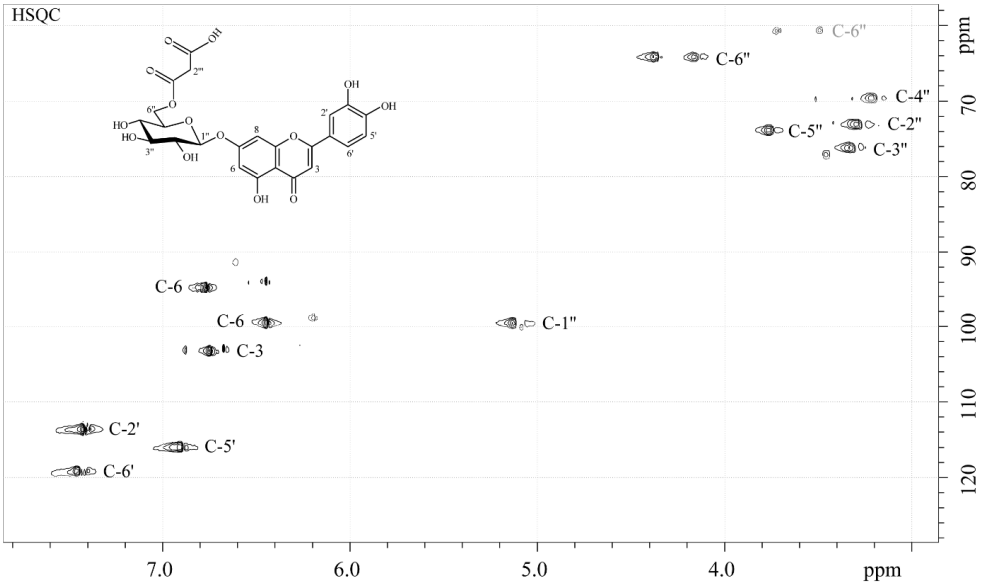


Figure F-6. HSQC spectrum of luteolin 7-*O*-β-(6"-*O*-malonyl)glucoside (**6**), dissolved in d_6-DMSO, recorded at 25°C.

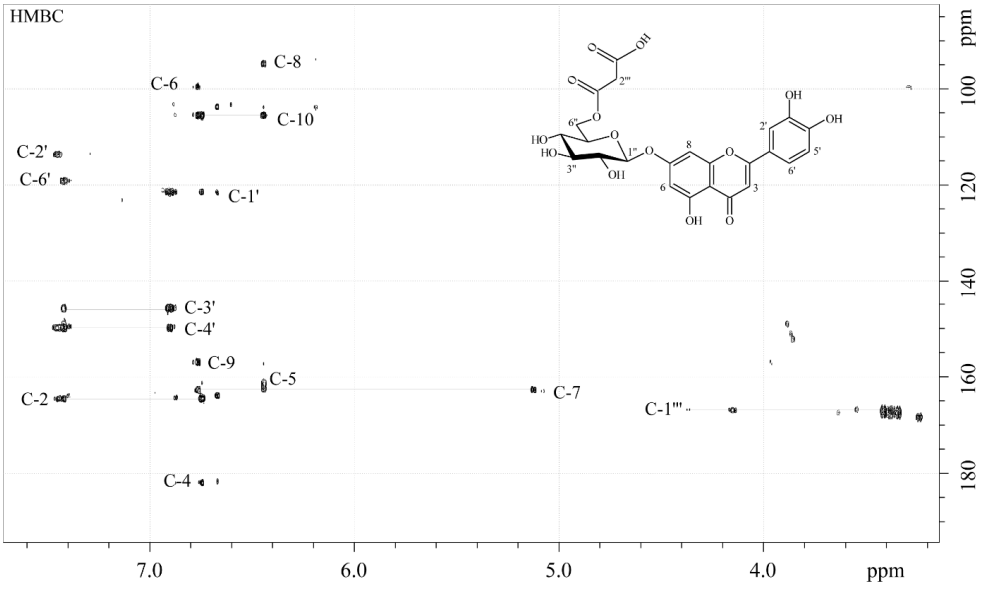


Figure F-7. HMBC spectrum of luteolin 7-*O*-β-(6"-*O*-malonyl)glucoside (**6**), dissolved in d_6-DMSO, recorded at 25°C.

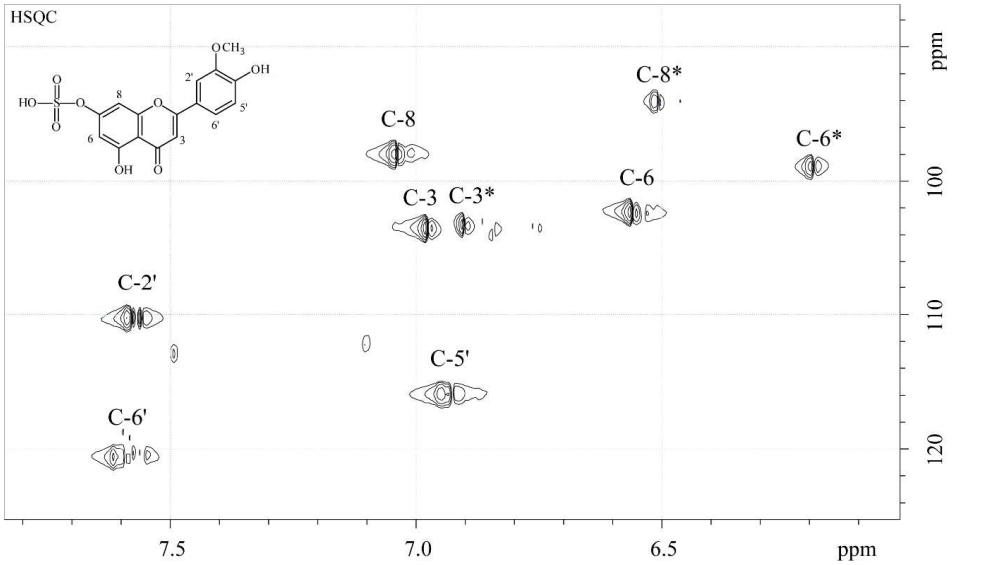


Figure F-8. HSQC spectrum of chrysoeriol 7-*O*-sulphate (**9**), dissolved in d_6-DMSO, recorded at 25°C. Crosspeaks marked with * are signals from chrysoeriol (**14**), due to loss of sulphate.

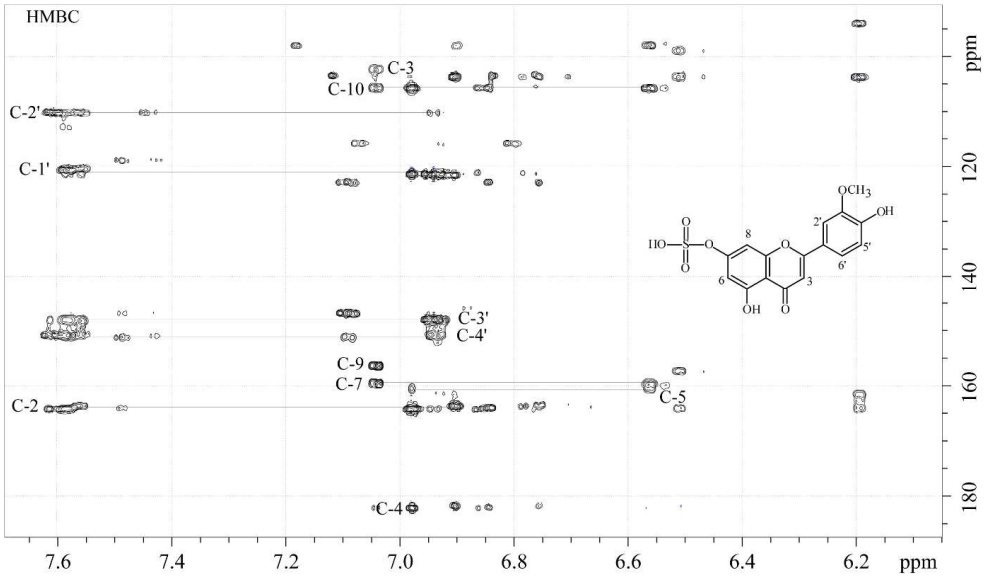


Figure F-9. HMBC spectrum of chrysoeriol 7-*O*-sulphate (**9**), dissolved in d_6-DMSO, recorded at 25°C.

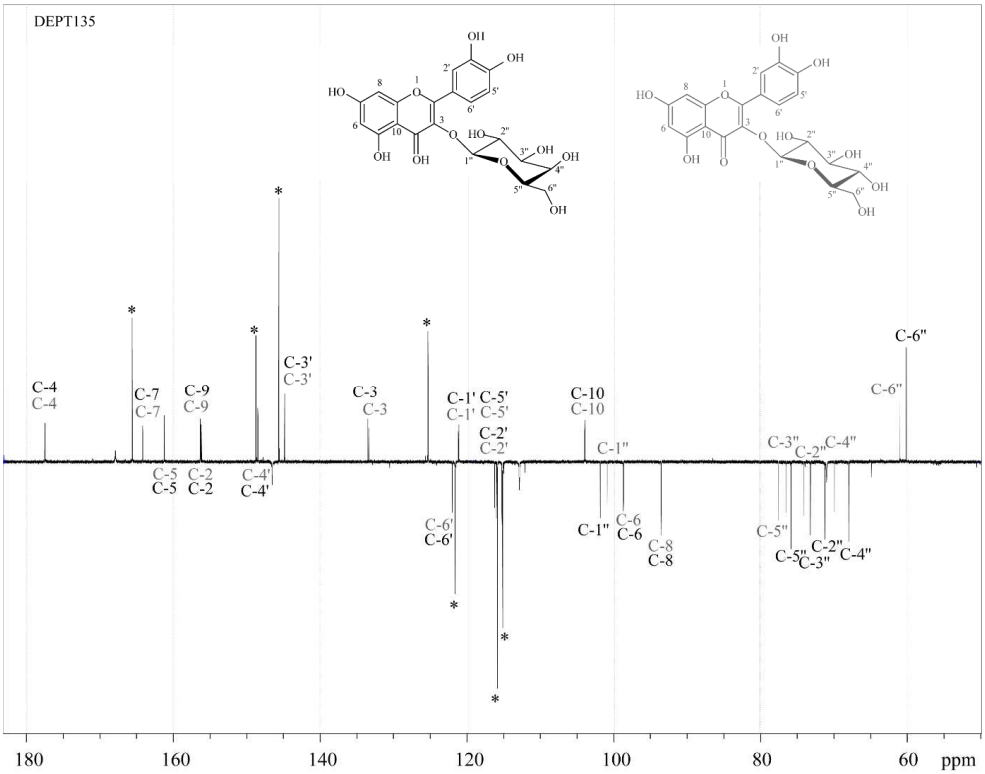


Figure F-10. ^{13}C (213.765 MHz) DEPT-135 spectrum of quercetin 3-*O*-galactoside (**16**) and quercetin 3-*O*-glucoside (**17**), dissolved in d_6-DMSO, recorded at 25°C. * signals from chicoric acid

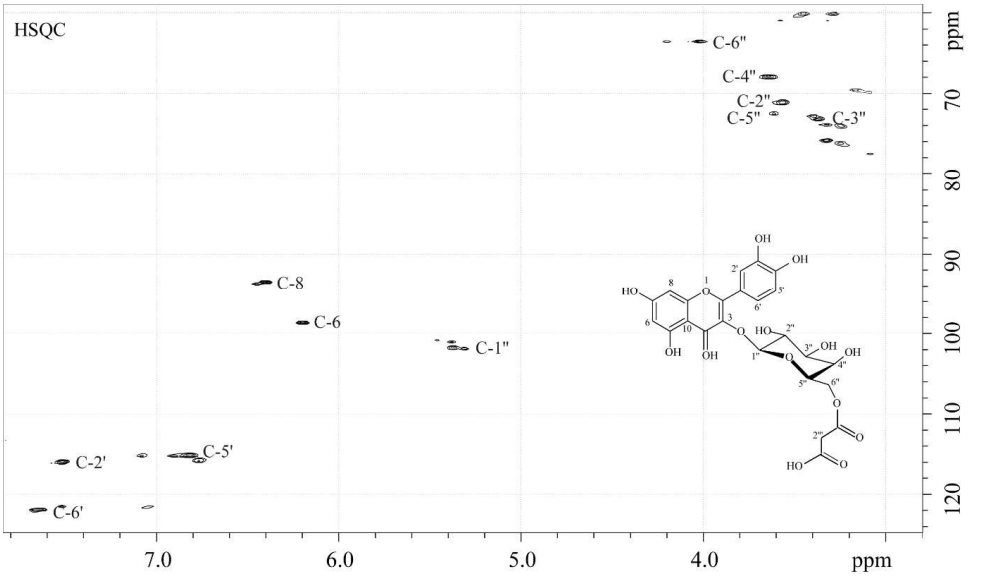


Figure F-11. HSQC spectrum of quercetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside (**19**), dissolved in d_6-DMSO, recorded at 25°C

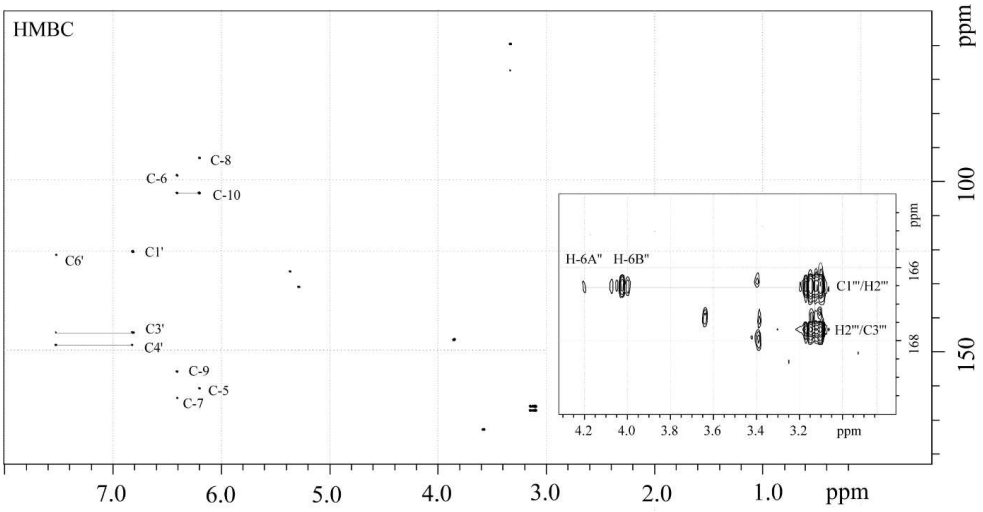


Figure F-12. HMBC spectrum of quercetin 3-*O*-β-(6"-*O*-malonyl)galactopyranoside (**19**), dissolved in d_6-DMSO, recorded at 25°C

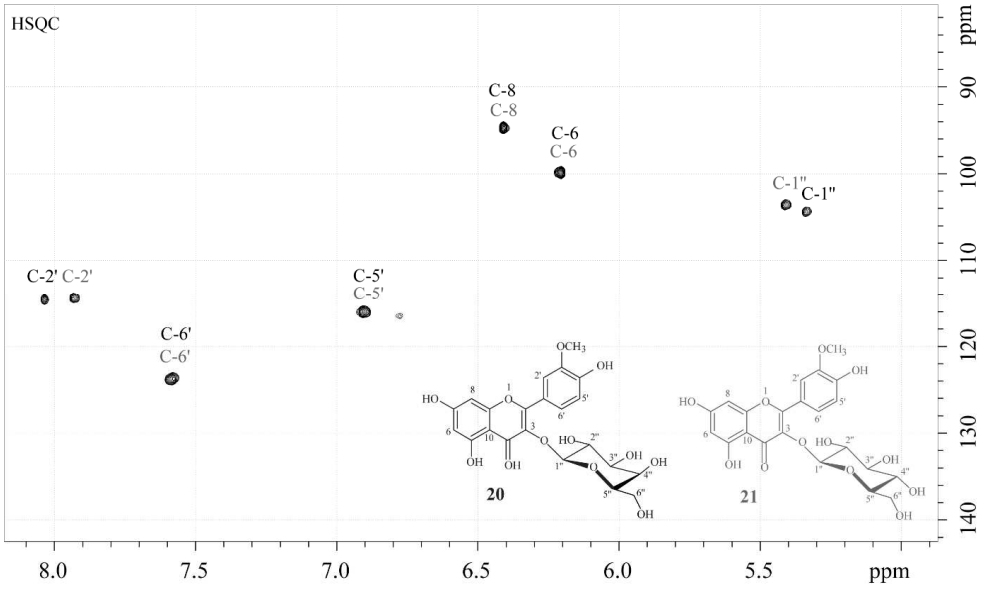


Figure F-13. HSQC spectrum of isorhamnetin 3-*O*-galactoside (**20**) and isorhamnetin 3-*O*-glucoside (**21**), dissolved in d_4-MeOD, recorded at 25°C.

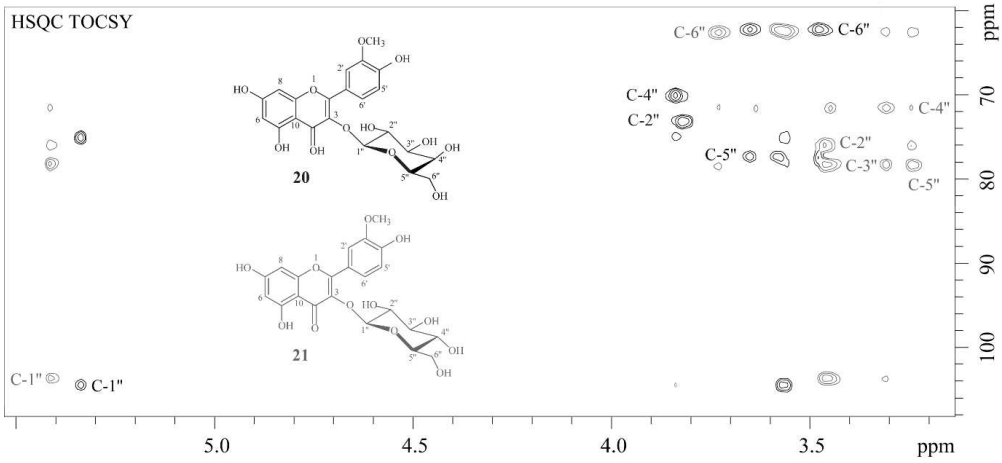


Figure F-14. HSQC-TOCSY spectrum of isorhamnetin 3-*O*-galactoside (**20**) and isorhamnetin 3-*O*-glucoside (**21**), dissolved in d_4-MeOD, recorded at 25°C.

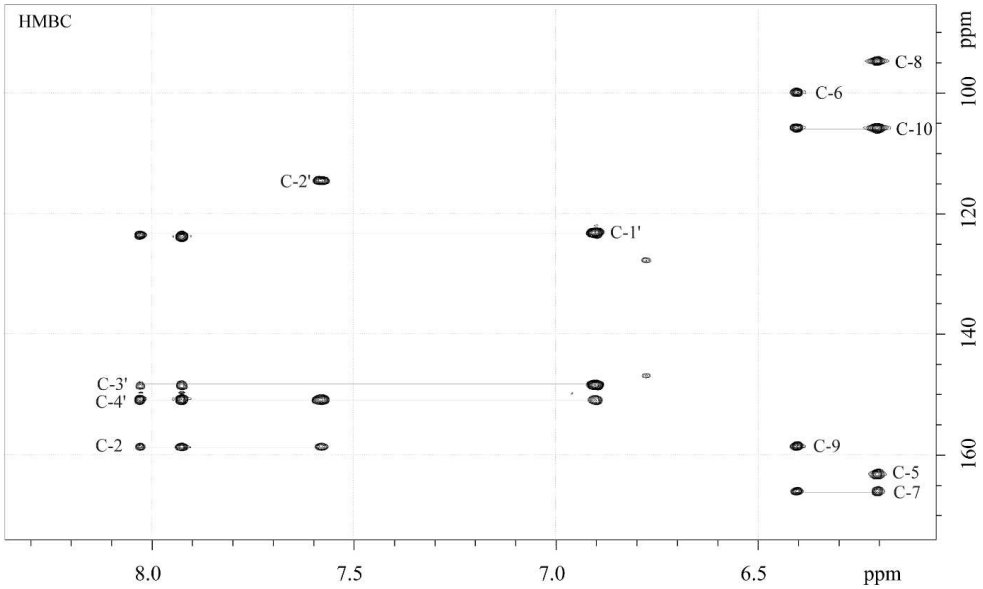


Figure F-15. HMBC spectrum of isorhamnetin 3-*O*-galactoside (**20**) and isorhamnetin 3-*O*-glucoside (**21**), dissolved in d_4-MeOD, recorded at 25°C.

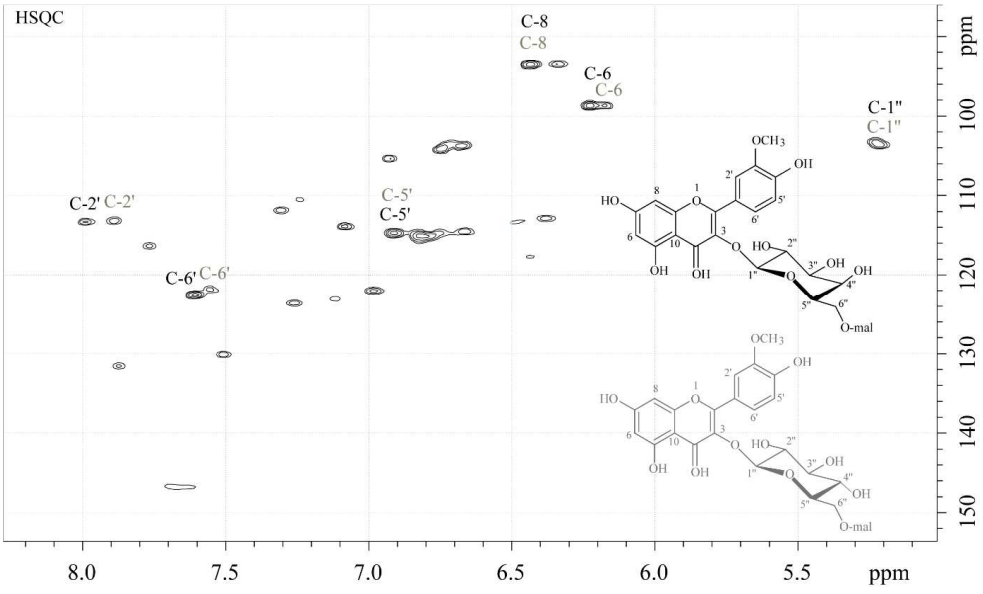


Figure F-16. HSQC spectrum of isorhamnetin 3-*O*-(6"-*O*-malonyl)galactoside (**22**) and isorhamnetin 3-*O*-(6"-*O*-malonyl)glucoside (**23**), dissolved in d_4-MeOD, recorded at 25°C.

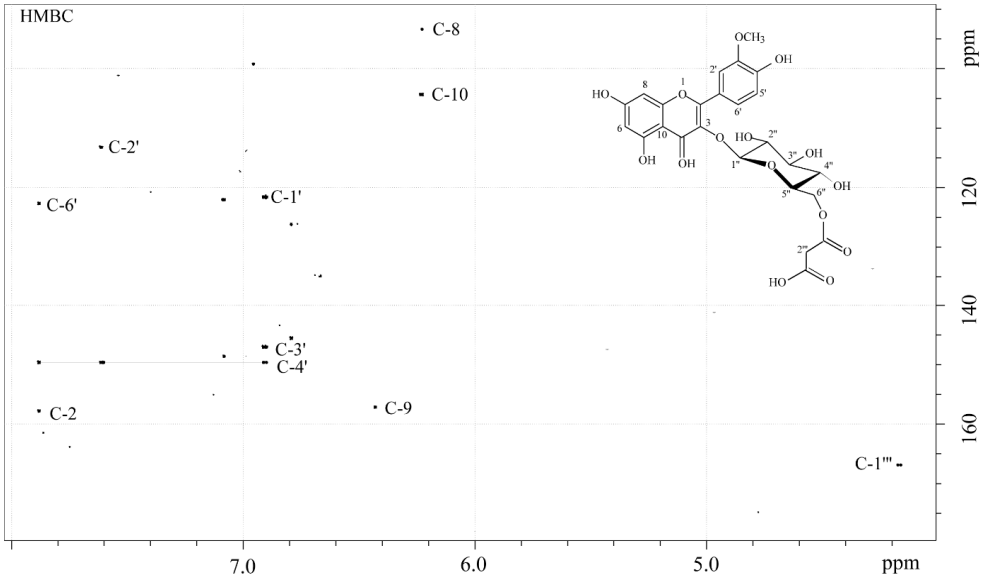


Figure F-17. HMBC spectrum of isorhamnetin 3-*O*-(6"-*O*-malonyl)glucoside (**23**), dissolved in d_4-MeOD, recorded at 25°C.

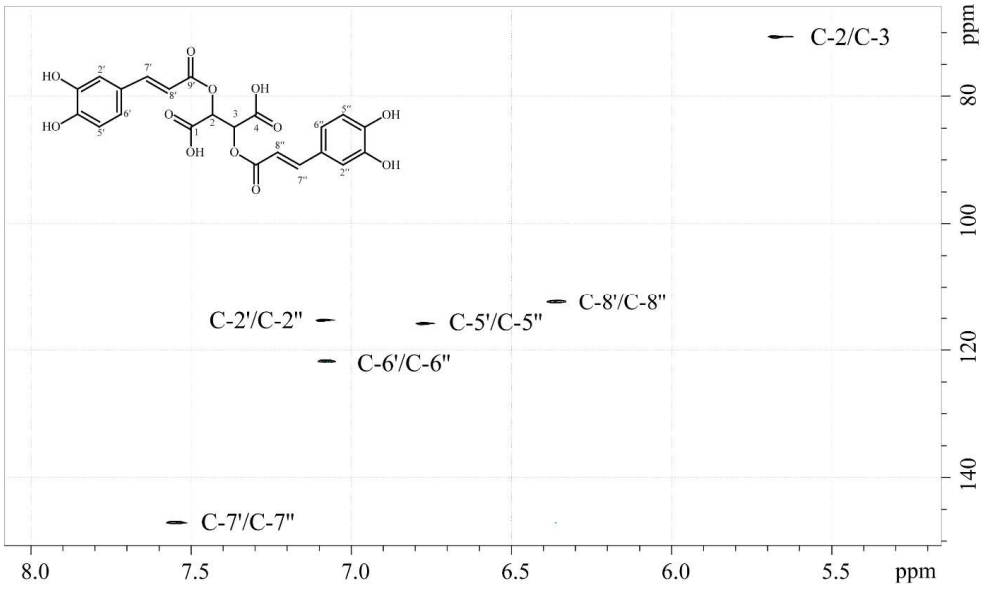


Figure F-18. HSQC spectrum of chicoric acid (CA) in d_6-DMSO, recorded at 25°C.

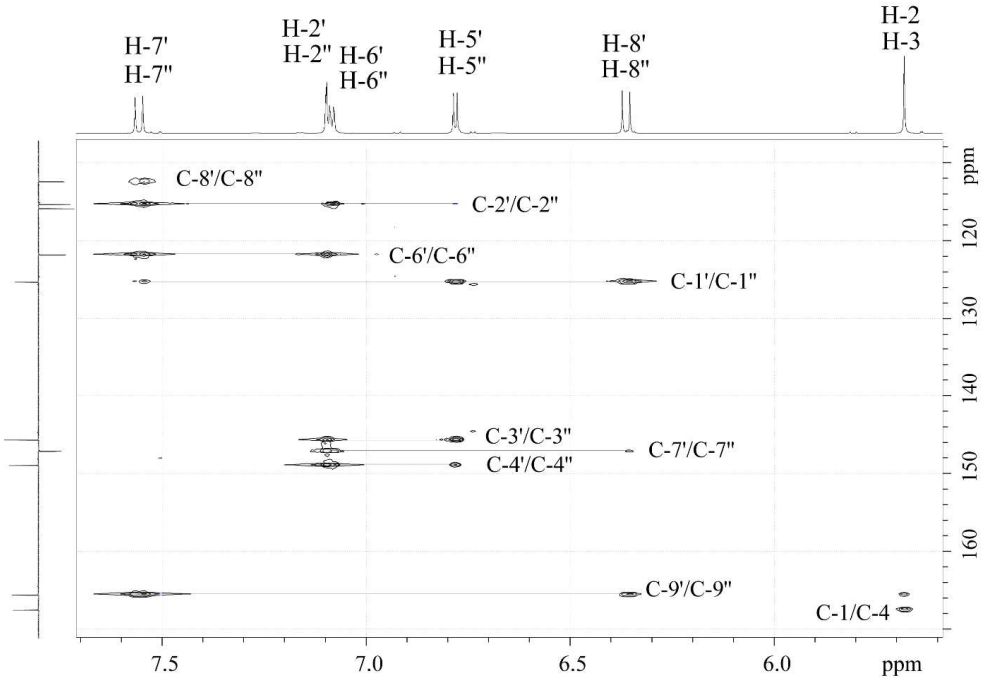


Figure F-19. HMBC spectrum of chicoric acid (CA) in d_6-DMSO, recorded at 25°C.

APPENDIX G – Quantitative data

Table G-1. Quantitative amounts[1] of individual flavonoids and rosmarinic acid (**RA**) in leaves of *Z. marina* collected from Espegrend (A) in 2014-2017.

	1	**2**	**3**	**4**	**6**	**7**	**RA**	**8**	**9**	**10**	**11**	**12**	**TF**
2014													
Apr	4.7 ± 0.2	1.0 ± 0.02	0.5 ± 0.1	3.4 ± 0.3	1.7 ± 0.2	0.5 ± 0.02	5.3 ± 0.9	0.3 ± 0.01	1.4 ± 0.03	7.4 ± 0.7	0.3 ± 0.03	0.6 ± 0.01	21.8 ± 1.4
June	4.5 ± 0.2	0.9 ± 0.08	0.4 ± 0.02	3.2 ± 0.3	0.9 ± 0.04	0.5 ± 0.01	6.1 ± 0.4	0.4 ± 0.01	2.2 ± 0.1	6.2 ± 0.7	0.3 ± 0.01	1.2 ± 0.08	20.6 ± 1.1
Sep	3.8 ± 0.3	0.8 ± 0.04	0.2 ± 0.02	1.0 ± 0.17	0.3 ± 0.05	0.4 ± 0.05	3.9 ± 0.7	0.3 ± 0.03	2.9 ± 0.3	2.0 ± 0.3	0.3 ± 0.03	1.3 ± 0.2	13.5 ± 1.3
2015													
May	4.7 ± 0.2	1.0 ± 0.01	0.8 ± 0.03	4.2 ± 0.5	2.0 ±0.1	0.4 ± 0.02	5.2 ± 0.2	0.3 ± 0.02	1.5 ± 0.1	8.4 ± 0.7	0.3 ± 0.001	0.9 ± 0.1	24.4 ± 1.4
June	4.0 ± 0.4	0.8 ± 0.1	0.7 ± 0.03	3.3 ± 0.3	1.3 ± 0.1	0.5 ± 0.01	6.9 ± 0.7	0.4 ± 0.01	2.5 ± 0.1	6.2 ± 0.7	0.3 ± 0.02	1.7 ± 0.2	21.6 ± 1.6
Sep	5.2 ± 0.5	1.1 ± 0.1	0.3 ± 0.04	2.1 ± 0.6	0.5 ± 0.1	0.5 ± 0.05	3.5 ± 0.7	0.3 ± 0.02	3.5 ± 0.8	3.1 ± 0.7	0.3 ± 0.02	1.6 ± 0.3	18.5 ± 2.8
2016													
May	3.8 ± 0.2	1.0 ± 0.03	0.7 ± 0.03	2.3 ± 0.3	1.5 ± 0.2	0.4 ± 0.02	3.6 ± 0.1	0.3 ± 0.03	1.8 ± 0.1	7.5 ± 0.8	0.4 ± 0.1	0.9 ± 0.03	20.6 ± 1.6
June	5.0 ± 0.4	0.9 ± 0.04	0.5 ± 0.04	3.7 ± 0.7	0.9 ± 0.1	0.4 ± 0.04	3.5 ± 0.4	0.3 ± 0.01	3.0 ± 0.3	7.9 ± 1.0	0.4 ± 0.1	1.6 ± 0.1	24.5 ± 2.6
Sep	5.3 ± 0.8	1.1 ± 0.1	0.2 ± 0.01	0.8 ± 0.1	0.3 ± 0.1	0.3 ± 0.03	1.1 ± 0.4	0.2 ± 0.01	2.4 ± 0.4	3.0 ± 0.3	0.3 ± 0.05	1.0 ± 0.1	15.0 ± 1.9
2017													
Feb	3.7 ± 0.4	1.1 ± 0.1	0.3 ± 0.1	0.9 ± 0.2	0.5 ± 0.1	0.3 ± 0.04	1.4 ± 0.3	0.2 ± 0.02	1.4 ± 0.2	4.1 ± 0.7	0.4 ± 0.04	0.6 ± 0.1	13.5 ± 1.8
Mar	4.0 ± 0.1	1.1 ± 0.1	0.4 ± 0.05	0.7 ± 0.1	0.7 ± 0.1	0.4 ± 0.04	1.0 ± 0.2	0.3 ± 0.03	1.6 ± 0.1	6.3 ± 0.4	0.5 ± 0.1	0.7 ± 0.04	17.1 ± 0.5

[1]amounts are expressed in mg/g (mean value ± SD, n=4) dry weigth, based on luteolin equivalents; [2] TF = total flavonoids

Table G-2. Quantitative amounts[1] of individual flavonoids and rosmarinic acid (**RA**) in leaves of *Z. noltii* from Huglo (D) collected in May, June, September (Sep) in 2016 and February (Feb) and March (Mar) 2017.

	3	**4**	**5**	**6**	**8**	**10**	**11**	**RA**	**TF**
2016									
May	1.8 ± 0.7	6.7 ± 1.5	0.6 ± 0.04	0.6 ± 0.1	0.9 ± 0.1	8.2 ± 0.8	0.8 ± 0.1	2.7 ± 1.9	19.7 ± 2.9
June	0.9 ± 0.2	8.5 ± 1.5	0.4 ± 0.1	0.8 ± 0.2	1.4 ± 0.2	4.8 ± 0.8	0.5 ± 0.1	3.4 ± 1.2	17.3 ± 2.9
Sep	1.3 ± 0.2	13.0 ± 3.5	0.4 ± 0.1	1.0 ± 0.1	1.7 ± 0.2	8.2 ± 1.2	0.6 ± 0.1	2.5 ± 0.3	26.2 ± 5.4
2017									
Feb	1.4 ± 0.1	9.7 ± 1.1	0.5 ± 0.04	1.5 ± 0.1	1.5 ±0.1	12.2 ±0.9	0.6 ± 0.04	2.8 ± 0.4	27.3 ± 2.3
Mar	1.6 ± 0.2	7.5 ± 1.3	0.5 ± 0.1	1.6 ± 0.2	1.0 ±0.1	9.7 ± 1.3	0.6 ± 0.1	2.3 ± 0.4	22.4 ± 3.2

[1]amounts are expressed in mg/g (mean value ± SD, n=4) dry weigth, based on luteolin equivalents; [2] TF = total flavonoids

APPENDIX H – Optimization of extraction

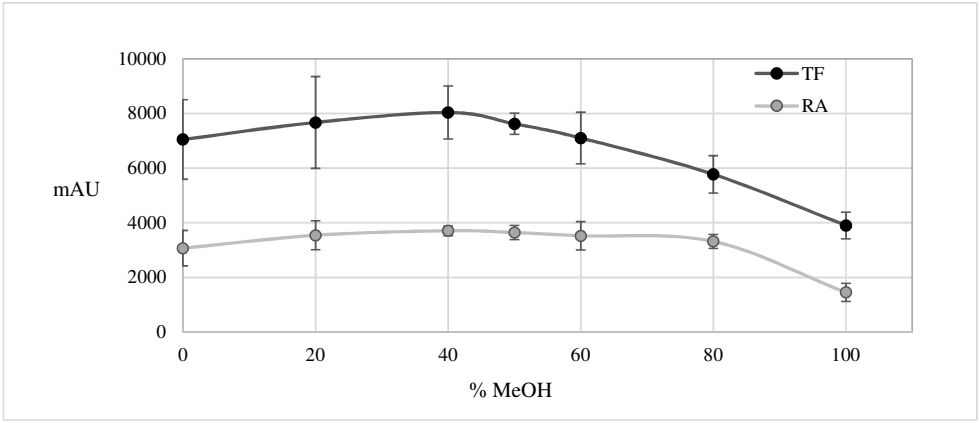


Figure H-1. Peak area of rosmarinic acid (**RA**) and total flavonoids (TF) measured at 360 ± 10 nm, at different concentrations of aqueous methanol (MeOH).

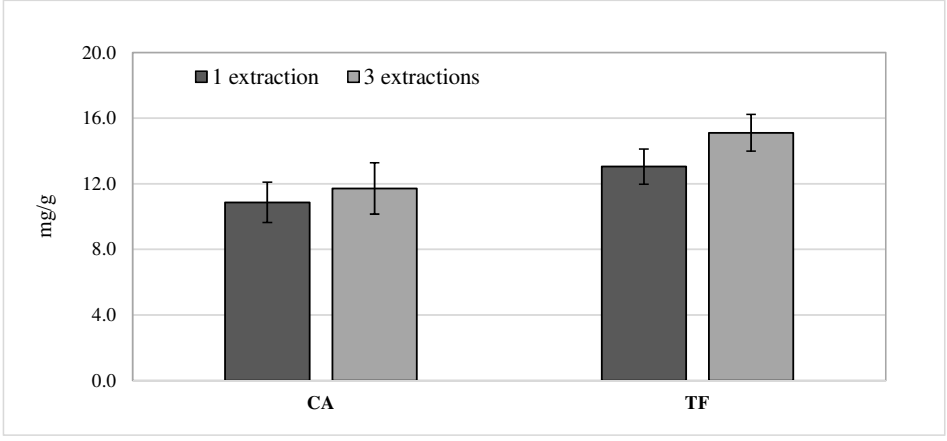


Figure H-2. Yield (in mg/g DW)) of chicoric acid (**CA**) and total flavonoids (TF) in *Ruppia cirrhosa* from 1 and 3 extractions. Mean value ± standard deviation (n=3).

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Isolation and Identification of Flavonoids Found in *Zostera marina* Collected in Norwegian Coastal Waters

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Abstract

In extracts of the seagrass *Zostera marina*, collected in coastal waters of West-Norway, fourteen different flavones and high amounts of rosmarinic acid were identified. Five of the flavones were found to be sulphated, among these were luteolin 7,3'-disulphate and chrysoeriol 7-sulphate structures previously not published with complete NMR assignments. Luteolin 7-*O*-β-(6''-malonyl) glucoside, and two other malonylated flavone compounds occurring in trace amounts, were identified for the first time in *Z. marina.* The sulphated flavones were fairly stable in slightly acidified (0.1% trifluoroacetic acid) extracts stored for months, however, under more acidic conditions (0.5% trifluoroacetic acid in the extracts) they were susceptible to undergo hydrolyses. When the solvents of purified fractions were removed by rotary evaporation, the sulphated flavones quickly decomposed to their corresponding aglycones due to the increased acid concentrations.

Keywords

***Zostera marina*, Sulphated, Flavones, NMR, Spectral Data, Characterization, Stability**

1. Introduction

Seagrasses are marine, rooted, flowering plants with terrestrial origin [1]. There are more than 70 species of seagrasses worldwide [2], but only four species of seagrasses have been found in European waters, namely *Zostera marina* L. (eelgrass), *Zostera noltii* (dwarf eelgrass), *Cymodoceanodosa* and *Posidoniaoceanica* [1]. Two of these: *Z. marina* and *Z. noltii*, are native to Norwegian coastal waters, in addition to *Z. angustifolia* which is considered as a variety of *Z. marina*. *Z. marina*, the most widely distributed seagrass in Norway, is most com-

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mon in the southern parts of Norway, but has also been found in the northern areas [3] [4]. The marine seagrasses form an ecological and therefore paraphyletic group of marine hydrophilus angiosperms which evolved three to four times from land plants towards an aquatic and marine existence [5]. Their taxonomy is not properly solved on the species level and below mainly due to their reduced morphology. Their physiology is also not well understood due to difficult experimental *in situ* and *in vitro* conditions. Seagrasses contain several compounds which make them different from terrestrial plants; some of these compounds might be of commercial interest. Harborne and Williams work back in the 70ties [6] revealed the occurrence of flavonoid sulphates in *Zostera* on the basis of TLC, electrophoretic mobility, λ_{max} and colour in UV light, and sulphated flavonoids were found to be more common in plants than previously considered [7]. So far, more than 150 sulphated flavonoids have been found in nature [8], most of which is based on flavones or flavonols. In plants, sulphated flavonoids are reported to be involved in regulation of plant growth [9]-[11], and they might form stable complexes with other flavonoids, for example anthocyanins [11]. It is also suggested that sulphation of flavonoids represents an ecological adaptation, due to the presence of sulphated flavonoids in numerous plants growing in marine habitats [9] [12]. Flavonoids are in general known for their wide range of biological activities [13]-[18] and several studies have addressed in particular sulphated flavonoids for their anticoagulant [9] [10], anti-inflammatory, antiviral and antitumor activities [11] [19]. Relevant here are some comparative studies of luteolin and luteolin 7,3'-disulphate from extracts of *Z. marina* [20] [21]. The disulphated flavone showed the highest pharmacological activities explained by its higher water solubility, which facilitated the absorption of the flavonoid in the intestines causing higher concentration of the flavonoid in the blood [20]. The sulphate ester bonds to flavonoids are, however, considered as relative unstable, implying that sulphated flavonoids [7] [12] might be degraded during extraction, purification and storage. After optimization of extraction and isolation conditions, addressing in particular the impact of solvent acidity on the unstable ester bonds in mono- and di- sulphated flavones, the flavonoid and rosmarinic content of *Z. marina* collected in Norwegian seawaters are here reported for the first time. Among the fourteen different flavones which were identified, five were found to be sulphated. Two of these have never been completely assigned with NMR data before. We also report on three flavones, which have not been identified previously in *Z. marina*.

2. Experimental

2.1. Plant Material

Zostera marina L. was collected during spring low tide by hand at a locality close to Espegrend Marine Biological Station outside Bergen, Norway. The sample locality (60˚16'12.0"N, 05˚13'20.3"E) was situated in a small sheltered bay, influenced by fresh water from a small brook. *Z. marina* formed a large patch growing in fine, muddy sediment. The collected material was washed thoroughly in fresh water and air-dried. The root was separated from the rest of the plant, and the material was cut in small pieces and stored at −20˚C, when not used. A voucher specimen has been deposited in the Herbarium BG at the University Museum of Bergen, Bergen.

2.2. Extraction and Purification

The seagrass was extracted 3 times with 50% aqueous methanol, after optimization of extraction conditions. The extracts were filtered through glass wool, and the methanol was removed using a rotary evaporator under reduced pressure at 27˚C, followed by partitioning with ethyl acetate. The aqueous layer, containing the flavonoids, was further concentrated and applied to an Amberlite XAD-7 column (70 × 5 cm, Sigma-Aldrich, Steinheim, Germany). The flavonoids were eluted with distilled water until the fractions were colorless, and then methanol was applied for elution of adsorbed flavonoids. Obtained fractions were analyzed by analytical HPLC-DAD, and fractions containing similar qualitative flavonoid content were combined and concentrated under reduced pressure. The semi-purified plant extract was submitted to preparative HPLC to obtain purified compounds. The purified fractions were evaporated under reduced pressure at 27˚C, and were further analyzed by HRLC-MS and NMR spectroscopy.

2.3. Stability Observations

Approximately 50 mg of dried Z. *marina* leaves was extracted with 50% methanol with 0.1%, 1.0% formic acid, 0.1% and 0.5% trifluoroacetic acid (TFA) for 1 hour at 25˚C. The extracts were filtered and analyzed periodi-

cally by analytical HPLC over 3 months period, and compared with a corresponding extract containing no acid. The relative content of sulphated flavonoids in the extract was determined by peak area measurement at 360 nm of individual compounds, relative to the total area of all flavonoids in the sample.

2.4. General Instrumentation

Analytical HPLC: The Agilent 1100 HPLC system was equipped with a HP 1050 diode array detector and a 200 × 4.6 mm inside diameter, 5 µm ODS Hypersil column (Supelco, Bellefonte, PA). Two solvents, (A) water (0.5% TFA) and (B) acetonitrile (0.5% TFA), were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by a linear gradient elution to 50% B. The flow rate was 1.0 mL/min, and aliquots of 15 µL were injected with an Agilent 1100 series microautosampler. The UV-Vis absorption spectra were recorded online during HPLC analysis over the wavelength range of 240 - 600 nm in steps of 2 nm. Preparative HPLC: The system used a Gilson 321 pump equipped with an Ultimate 3000 variable wavelength detector, a 25 × 2.2 cm (10 µm) Econosphere C18 column (Grace, Deerfield, IL), and the solvents (A) water (0.1% formic acid) and (B) acetonitrile (0.1% formic acid). Following gradient was used: 0 - 5 min; 15% - 20% B, 5 - 25 min; 20% - 30% B, 25 - 28 min; 30% - 40% B, 28 - 30 min 40% - 15% B. The flow rate was 15 mL/min. NMR-spectroscopy: One-dimensional ^{1}H, 2D heteronuclear single quantum coherence (^{1}H-^{13}C HSQC), heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC), double quantum filtered correlation (^{1}H-^{1}H DQF COSY) and total correlation spectroscopy (^{1}H-^{1}H TOCSY) experiments were obtained on a Bruker 600 MHz instrument equipped with a cryogenic probe. Sample temperatures were stabilized at 298 K. The deuteriomethyl^{13}C signal and the residual ^{1}H signal of the solvent (d_6-DMSO) were used as secondary references (δ 39.5 and 2.5 from TMS, respectively).High-resolution LC-electrospray mass spectrometry (ESI^{+}/TOF), spectra were recorded using a JEOL AccuTOF JMS-T100LC in combination with an Agilent Technologies 1200 Series HPLC system at the following instrumental settings/conditions; Ionization mode: positive, ion source temperature = 250˚C, needle voltage = 2000 V, desolvation gas flow = 2.0 L/min, nebulizing gas flow = 1.0 L/min, orifice1 temperature = 100˚C, orifice2 voltage = 6 V, ring lens voltage = 18 V, ion guide peak voltage = 2000 V, detector voltage = 2300 V, acquisition range = 100 - 1000 m/z, spectral recording interval = 0.5 s, wait time = 0.03 ns and data sampling interval = 0.5 ns. Sample was solved in a mixture of water and acetonitrile with 0.1% formic or acetic acid. The elution profile for HPLC consisted of initial conditions with 90% A (water with 0.1% formic acid) and 10% B (acetonitrile with 0.1% formic acid), isocratic elution 0 - 2 min, followed by a linear gradient elution to 50% B (2 - 15 min). A 50 × 4.6 mm internal diameter, 1.8 µm Agilent Zorbax Eclipse XDB C18 column was used for separation.

3. Results and Discussion

3.1. Characterization of *Zostera marina* Flavones

The HPLC profile of *Zostera marina* extract (**Figure 1**) revealed the presence of three major (**1**, **4**, **8**) and five minor flavones (**2**, **3**, **5**, 7, **9**) (**Figure 2** and **Table 1**), together with higher amounts of rosmarinic acid (**6**). In addition, traces of six flavones (**10**-**15**) were found during HRLC-MS examinations of the extracts. Five of these flavones (**1**, **2**, **4**, **7**, **8**) were substituted with sulphate groups, and the order of retention times in the HPLC reversed phase column system was found to be: disulphate (**1**) < monoglucoside (**3**) < monosulphate (**4**) < acyl glucoside (**5**) < aglycone (**9**), here exemplified with luteolin derivatives.

As shown in **Figure 3** the UV absorption spectra of luteolin 7-sulphate (**4**) and luteolin (**9**) are relative similar, and their UV_{max} values are consistent with previously reported data for flavones and flavone glycosides [22], whilst the significant hypsochromic shift in the UV_{max} of luteolin 7,3'-disulphate (**1**), is strongly indicating the presence of a sulphate group in the 3'- or 4'-position on the B-ring. Thus, introducing a sulphate group to the flavonoid A-ring, does not influence the UV absorption significantly, but sulphation in the 3'- or 4'-position on the B-ring will cause a large hypsochromic shift in band I. Thus flavonoid sulphates seem to have analogous UV spectral characteristics as their corresponding flavonoid glycosides [12].

3.2. Stability of Sulphated Flavones

The stability of the sulphated flavones in *Z. marina* extracts was investigated under various acidic conditions. The compounds were quite stable in extracts containing 0.1% - 1.0% formic acid and in 0.1% TFA, and did not

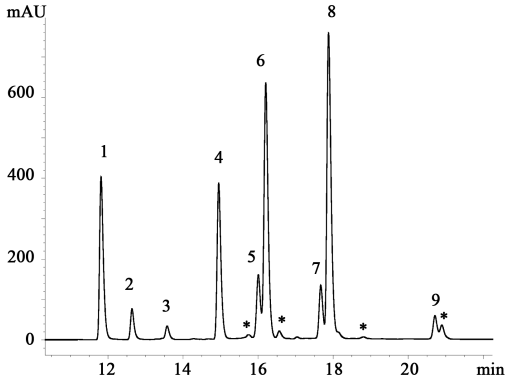
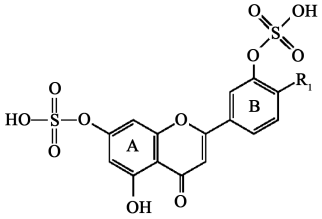
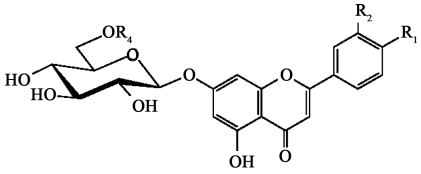


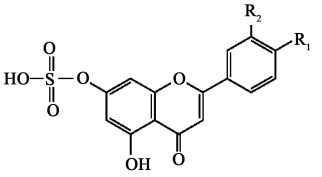
Figure 1. HPLC chromatogram of *Zostera marina* extract (recorded at 360 nm). **1** = luteolin 7,3'-disulphate, **2** = diosmetin 7,3'-disulphate, **3** = luteolin 7-*O*-β-glucoside, **4** = luteolin 7-sulphate, **5** = luteolin 7-*O*-β-(6"-malonyl)glucoside, **6** = rosmarinic acid, **7** = chrysoeriol 7-sulphate, **8** = diosmetin 7-sulphate, **9** = luteolin, *unidentified compounds.



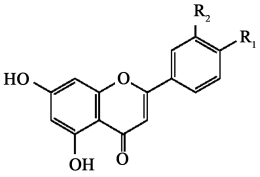
1 R_1 = OH
2 R_1 = OCH_3



3 R_1 = R_2 = OH, R_4 = H
5 R_1 = R_2 = OH, R_4 = malonyl
10 R_1 = OH, R_2 = R_4 = H
11 R_1 = OH, R_2 = H, R_4 = malonyl
12 R_1 = OH *or* OCH_3, R_2 = OCH_3 *or* OH, R_4 = malonyl



4 R_1 = R_2 = OH,
7 R_1 = OH, R_2 = OCH_3
8 R_1 = OCH_3, R_2 = OH



9 R_1 = OH = R_2 = OH,
13 R_1 = OH, R_2 = OCH_3
14 R_1 = OCH_3, R_2 = OH
15 R_1 = OCH_3, R_2 = OH

Figure 2. Structures of the flavones found in *Zostera marina* leaves. **1** = luteolin 7,3'-disulphate, **2** = diosmetin 7,3'-disulphate, **3** = luteolin 7-*O*-β-glucoside, **4** = luteolin 7-sulphate, **5** = luteolin 7-*O*-β-(6"-malonyl)glucoside, **7** = chrysoeriol 7-sulphate, **8** = diosmetin 7-sulphate, **9** = luteolin, **10** = apigenin 7-glucoside, **11** = apigenin 7-(6"-malonyl)glucoside, **12** = diosmetin- *or* chrysoeriol 7-(6"-malonyl)glucoside, **13** = apigenin, **14** = chrysoeriol, **15** = diosmetin. The flavones **10**-**15** are only present in trace amounts in the plant extract.

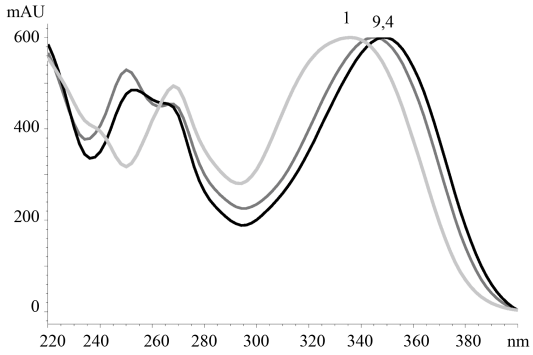


Figure 3. UV absorbance spectra for **1** (luteolin 7,3'-disulphate), **4** (luteolin 7-sulphate) and **9** (luteolin).

Table 1. Chromatographic and spectral (UV-vis and MS) data of the flavones and rosmarinic acid (**6**) in *Zostera marina*.

Compound	Online HPLC			LC-MS			Molecular formula
	UV_{max} (nm)	Local UV_{max} (nm)	t_R (min)	$[M + 1]^+$ m/z (observed)	Fragment m/z	$[M + 1]^+$ m/z (calculated)	
1	337	267	11.86	446.9725	367.0143, 287.0578	446.9692	$C_{15}H_{10}O_{12}S_2$
2	333	269	12.70	460.9869	381.0276, 301.0693	460.9848	$C_{16}H_{12}O_{12}S_2$
3	348	253, 266	13.53	449.1086	287.0562	449.1084	$C_{21}H_{20}O_{11}$
4	349	253, 266	14.96	367.0127	287.0564	367.0124	$C_{15}H_{10}O_9S$
5	338	252, 266	15.96	535.1080	-	535.1088	$C_{24}H_{22}O_{14}$
6	330	290 (sh)	16.18	361.0929	163.0386	361.0923	$C_{18}H_{16}O_8$
7	348	252, 266	17.69	381.0283	301.0719	381.0280	$C_{16}H_{12}O_9S$
8	347	252, 266	17.91	381.0283	301.0719	381.0280	$C_{16}H_{12}O_9S$
9	346	250, 268	20.77	287.0553	-	287.0556	$C_{15}H_{10}O_6$
10*				433.1140			$C_{21}H_{20}O_{10}$
11*				519.1155			$C_{24}H_{22}O_{13}$
12*				549.1242			$C_{25}H_{24}O_{14}$
13*				271.0605			$C_{15}H_{11}O_5$
14*				301.0701			$C_{16}H_{12}O_6$
15*				301.0701			$C_{16}H_{12}O_6$

sh = shoulder. *only found in trace amounts in extracts (**10**: apigenin 7-glucoside, **11**: apigenin **7**: (malonyl)glucoside, **12**: diosmetin- or chrysoeriol **7**: (malonyl)glucoside, **13**: apigenin, **14**: chrysoeriol, **15**: diosmetin).

show significant differences when compared to their storage in the corresponding neutral methanolic extract for 3 months. However, in the extract containing 0.5% TFA, the flavone sulphates (**1**, **2**, **4**, **7** and **8**) decomposed gradually to their corresponding aglycones (**9**, **14** and **15**) due to acid hydrolysis. The sulphated flavones were isolated and purified by preparative HPLC, and their stability in the eluate solvent (consisting of water and acetonitrile with 0.1% formic acid) were monitored by analytical HPLC. The results showed that the sulphated flavones were relative stable in this solvent with a decay of 1% - 5% in the course of 10 days. However, when the solvent was removed by evaporation, these compounds quickly decomposed to their corresponding aglycones, due to accumulated acid concentrations. Despite the problems with instability of the sulphated flavones, we were able to obtain pure samples of **1** (14 mg), **4** (4 mg) and **7** (6 mg).

3.3. NMR Assignment of Luteolin 7,3'-Disulphate (1), Chrysoeriol 7-Sulphate (7) and Luteolin 7-*O*-β-(6''-Malonyl)Glucoside (5)

The ^{1}H NMR spectrum of compound **1** (Figure 2) showed six proton signals in the aromatic region; a pair of *meta* coupled protons at δ 6.57 (1 H, *d*, J = 2.06 Hz, H-6) and δ 6.98 (1 H, *d*, J = 2.01 Hz, H-8), a one proton singlet at δ 6.74 (H-3), and the AMX system at δ 6.99 (1 H, *d*, J = 8.3 Hz, H-5'), δ 7.93 (1 H, *d*, J = 2.34 Hz, H-2'), δ 7.71 (1 H, *d*, J = 2.35, 8.7 Hz, H-6'), which were in accordance with a luteolin derivative [23]. The ^{13}C NMR values for compound **1** (Table 2) were assigned on the basis of $^1J_{CH}$, $^2J_{CH}$, $^3J_{CH}$ and $^4J_{CH}$ correlations observed in the HSQC and HMBC spectra. The downfield carbon data for C-6, C-8 as well as the significantly downfield shifts of H-6 and H-8 strongly indicated the presence of an electron withdrawing sulphate ester in position C-7. Similarly, a second sulphate group was indicated by the NMR values of the protons H-2', H-5' and

Table 2. ^{1}H (600.13 MHz) and ^{13}C (150.90 MHz) NMR data for luteolin 7,3'-disulphate (**1**), luteolin 7-*O*-β-(6''-malonyl)glucoside (**5**) and chrysoeriol 7-sulphate (**7**), isolated from *Zostera marina* leaves. Compounds were dissolved in d_6-DMSO at 25˚C.

	1			**7**			**5**		
	^{13}C	^{1}H	J (*Hz*)	^{13}C	^{1}H	J (*Hz*)	^{13}C	^{1}H	J (*Hz*)
2	164.0			164.0			164.3		
3	103.0	6.74	*s*	103.4	6.98	*s*	103.1	6.74	*s*
4	181.9			182.0			181.8		
5	160.4			160.3			161.6		
6	102.2	6.57	*d* (2.1)	102.3	6.56	*d* (2.1)	99.30	6.44	*d* (2.1)
7	159.3			159.6			162.5		
8	97.7	6.98	*d* (2.0)	97.9	7.04	*d* (2.1)	94.6	6.76	*d* (2.2)
9	156.1			156.3			156.8		
10	105.4			105.7			105.3		
1'	120.3			121.4			121.3		
2'	120.3	7.93	*d* (2.3)	110.2	7.58	*d* (2.1)	113.5	7.42	*d* (2.2)
3'	141.3			147.7			145.9		
4'	153.7			150.6			146.9		
5'	117.3	6.99	*d* (8.3)	115.6	6.94	*d* (8.3)	115.9	6.90	*d* (8.4)
6'	123.4	7.71	*dd* (8.7, 2.3)	120.4	7.60	*dd* (8.3, 2.2)	119.0	7.45	*dd* (8.4, 2.2)
OCH_3				56.0	3.89				
Sugar									
1''							101.3	5.12	*d* (7.6)
2''							74.6	3.59	*m*
3''							77.7	3.58	*m*
4''							71.1	3.47	*dd* (8.7, 9.4)
5''							75.5	3.86	*m*
6A''							65.3	4.61	*dd* (2.1, 11.7)
6B''							65.3	4.41	*dd* (6.6, 11.7)
Acyl									
1'''							168.5		
2A'''							52.7	3.71	*s*
2B'''							52.7	3.81	*s*
3'''							169.2		

H-6', and the carbons C-2', C-5' and C-6', which were significantly shifted downfield when compared to the corresponding proton and carbon signals of luteolin. When compared to the carbon and proton values in luteolin 7,4'-disulphate [24], the same pattern can be seem, however in compound **1**, the protons and carbons in the 1', 3' and 5'-position were shifted downfield, due to the sulphate ester group in position 3'. Compound **1** is therefore identified as luteolin 7,3'-disulphate. The high resolution mass spectrum of **1** showed a positive molecular ion $[M+H]^+$ at m/z 446.9725 (**Table 1**), which confirms the identity of **1**. The observed fragments at m/z 367.0143 and 287.0578 indicating loss of one and two sulphate groups, were in accordance with luteolin 7-sulphate and luteolin, respectively.

The ^{1}H NMR spectrum of compound **7** showed signals for a pair of *meta* coupled protons at δ 6.56 (1 H, *d*, J = 2.06 Hz, H-6) and δ 7.04 (1 H, *d*, J = 2.09 Hz, H-8), a one proton doublet at δ 6.94 (1 H, *d*, J = 8.29 Hz, H-5'), a one proton doublet at δ 7.58 (1 H, *d*, J= 2.07 Hz, H-2'), a one proton double doublet at δ 7.60 (1 H, *dd*, J = 2.14, 8.28 Hz, H-6'), a one proton singlet at δ 6.98 (H-3), and a methoxy group at δ 3.89, corresponding to a diosmetin derivative [25]. The downfield shifts of protons H-6 and H-8 and carbon C-6 and C-8 were indicating a sulphate ester linked to the 7-position. The NMR data (**Table 2**) were partially in accordance with previously published NMR data on diosmetin 7-sulphate [25], but whereas the methoxy group on the B ring is in the C-4' position in diosmetin, the HMBC spectrum of compound **7** showed a long-range correlation between the methoxy protons (δ 3.89) and C-3' (δ147.7), which verified that the methoxy group was in the C-3' position. This means that the identity of compound **7** is chrysoeriol 7-sulphate, which was confirmed by HRLC-MS results showing a $[M+H]^+$ at m/z 381.0283 and a fragment at m/z 301.0719, corresponding to chrysoeriol 7-sulphate and chrysoeriol, respectively. Luteolin 7,3'-disulphate (**1**) and chrysoeriol 7-sulphate (**7**) have as far as we know only been reported to be in *Z. marina* previously [6], but these compounds have not been completely assigned with NMR data before.

The ^{1}H NMR spectrum of compound **5** showed six proton signals in the aromatic region; δ 6.44 (1 H, *d*, J = 2.10 Hz, H-6) and δ 6.76 (1 H, d, J = 2.18 Hz, H-8), a one proton singlet at δ 6.74 (H-3), and an AMX system at δ 6.90 (1 H, *d*, J = 8.36 Hz, H-5'), δ 7.42 (1 H, *d*, J = 2.20 Hz, H-2'), δ 7.45 (1 H, *dd*, J = 2.20, 8.38 Hz, H-6'), consistent with a luteolin derivative [23].The sugar region of **5** showed the presence of one unit. The ^{1}H and ^{13}C values of this sugar unit were assigned by a combination of 1D ^{1}H NMR, 2D COSY, TOCSY and HSQC experiments. The ^{1}H and ^{13}C resonances were in accordance with β-glucopyranose [26]. A long range coupling between the C-7 of the aglycone and the anomeric proton of the glucose unit confirmed the site of glucosylation to be at the 7-hydroxyl. The downfield shift values of H-6A" (δ 4.61) and H-6B" (δ 4.39) indicated acylation of the 6"-hydroxyl, and a long range coupling between the H-6" protons of the sugar and a carbonyl carbon (C-1'") at δ 168.5 (C-1'") was observed. Furthermore, there was a cross peak at δ 3.71/168.5 (H-2A'"/C-1'") and δ 3.81/169.2 (H-2B'"/C-3'") in the HMBC spectrum, corresponding to a malonyl unit. The molecular ion $[M+H]^+$ at m/z 535.1080 in the HRLC-MS of compound **5** confirmed the identity to be luteolin 7-*O*-β-(6"-malonyl) glucopyranoside. Luteolin 7-*O*-β-(6"-malonyl)glucopyranoside has been identified in terrestrial plants previously [27] [28], but this is the first time it has been reported in *Z. marina*. Malonylated flavone glucosides have just recently been reported to occur in marine environments [29].

3.4. NMR Characteristics of Sulphated Flavones

Despite the lack of NMR resonances of the sulphate moieties in sulphated flavonoids in ^{1}H and ^{13}C NMR spectra, the linkage position of potential sulphate groups might be revealed by comparison of their spectra with spectra of their non-sulphated analogs (**Table 3**). As described previously [12], protons and carbons in *orto* and *para* positions to the sulphate ester have higher chemical shift values than their corresponding protons and carbons of the aglycone, due to decreased shielding, whereas the carbon directly attached to the sulphate ester and the carbons in *meta* position have lower chemical shifts, due to increased shielding from the electron withdrawing sulphate ester. Both protons and carbons in positions 6, 8 and 10 on the A-ring in **1**, **4** and **7** have significantly higher chemical shifts than in their corresponding aglycones (**Table 3**), which confirm that **1**, **4** and **7** have a sulphate group connected to C-7. The HSQC spectrum of **4** is presented in **Figure 4**, and illustrates the downfield shifts of C-6 and C-8 as a result of introducing a sulphate group onto the A-ring. The carbon signals of the sulphated flavones are displayed in black, whereas the signals of the corresponding aglycone, due to loss of sulphate group, are displayed in grey.

Similar NMR shift effects were revealed caused by sulphation of the B-ring of **1**. A significant increase in the

Table 3. Diagnostic ^{13}C and ^{1}H NMR sulphation shifts[1] from spectra of luteolin 7,3'-disulphate (**1**), luteolin 7-sulphate (**4**) and chrysoeriol 7-sulphate (**8**).

Position of sulphation									
^{13}C	7 (**1**, **4**, **7**)		3' (**1**)		^{1}H	7 (**1**, **4**, **7**)		3' (**1**)	
Ipso	−4.4 to −4.7	(C-7)	−4.5	(C-3')	*Ipso*				
Orto	+3.4 to +3.6	(C-6)	+7.1	(C-2')	*Orto*	+0.3 to +0.4	(H-6)	+0.5	(H-2')
	+3.6 to +3.9	(C-8)	+4.0	(C-4')		+0.5 to +0.6	(H-8)	-	
Meta	−1.0 to −2.2	(C-5)	−0.7	(C-1')	*Meta*			+0.1	(H-5')
	−0.8 to −1.1	(C-9)	+0.6	(C-5')					
Para	+1.9 to +2.1	(C-10)	+4.3	(C-6')	*Para*			+0.3	(H-6')

[1]δ (sulphated flavonoid)-δ (aglycone) (in ppm).

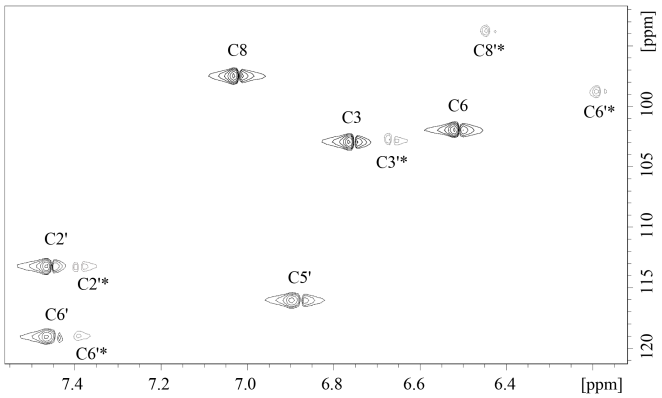


Figure 4. HSQC spectrum of compound **4** (luteolin 7-sulphate) in a mixture with **9** (luteolin). The cross peaks from compound **9** due to decomposing of **4** are shown in grey and are assigned with*.

shift values of C-2', C-4' and C-6', as well as a decrease in the chemical shift value of C-3' were observed, due to a sulphate ester group in the 3'-position on the B-ring. The increase in the chemical shift values of H-2' and H-6' were also in accordance with a sulphate ester in the 3'-position. The sulphate group induced shifts in 7-sulphate and 7,3'-disulphate of luteolin are illustrated in **Figure 5**, in which ^{1}H NMR spectra of both mono- and disulphated luteolin and luteolin are displayed. The instability of sulphated flavonoids becomes evident in this illustration, as signals corresponding to luteolin can be seen in the spectrum of luteolin 7-sulphate (**4**), due to loss of the sulphate group. It is also possible to see some weak proton signals of luteolin 7,3'-disulphate in the spectrum of luteolin (**9**), which is due to the fact that the original NMR sample contained exclusively the disulphate, yet the disulphate rapidly decomposed to luteolin during concentration of the sample.

4. Conclusion

After optimization of extractions conditions two sulphated flavones: luteolin 7,3'-disulphate and chrysoeriol 7-sulphate were isolated and identified on the basis of NMR and high resolution mass spectra data as well as hydrolysis studies. This is the first report with complete NMR data for these two compounds, and chemical shift variations created by the sulphate groups were observed. All the sulphated flavones found in *Zostera marina* were shown to be easily hydrolyzed during extraction, isolation and examination unless careful handling was performed. Luteolin 7-*O*-β-(6"-malonyl)glucoside was isolated and identified for the first time in *Z. marina*. Trace amounts of the malonylated flavone glucosides of apigenin and chryseriol/diosmetin previously not reported in *Z. marina*, were found in extracts by HRLC-MS.

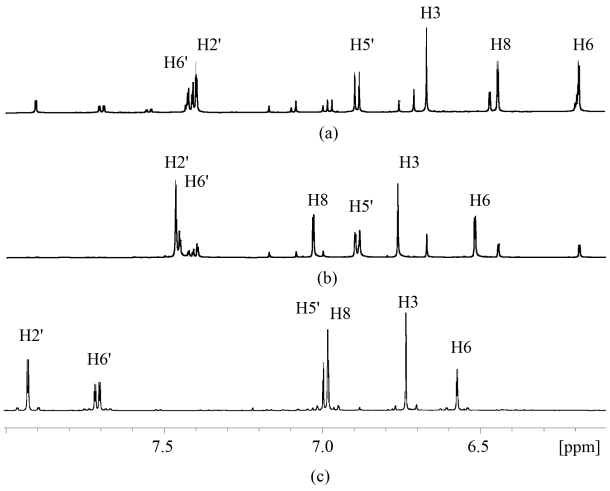


Figure 5. (a) ^{1}H NMR spectrum of **9** (luteolin), (b) ^{1}H NMR spectrum of **4** (luteolin 7-sulphate), (c) ^{1}H NMR spectrum of **1** (luteolin 7,3'-disulphate).

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Characterization and seasonal variation of individual flavonoids in *Zostera marina* and *Zostera noltii* from Norwegian coastal waters

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ABSTRACT

The flavonoid content in leaves of *Zostera marina* and the endangered *Zostera noltii*, including mono- and disulphated flavonoids, from different sample localities were characterized. Seasonal variation of both individual and total flavonoid, as well as rosmarinic acid concentration were revealed. Minor amounts of luteolin 7-(6″-malonyl)glucoside (**6**) and apigenin7-(6″-malonyl)glucoside (**11**) were identified in *Z. noltii* for the first time. The total flavonoid content was found to be higher in *Z. noltii* than in *Z. marina* at most of the examined localities, and the qualitative flavonoid content was somewhat different in the two species. The quantitative variation of flavonoids and rosmarinic acid was found to be relatively consistent from year to year in *Z. marina* during a period of three years. The two species appeared though to have a different flavonoid production in the various seasons at the West coast. While *Z. marina* had the highest content in young leaves in May or June, with a markedly decrease from June to September and the lowest measured content in February, *Z. noltii* had the lowest measured flavonoid content in May/June followed by an increase from June to September and the highest measured content during wintertime in February. The observed seasonal differences may be related to the fact that *Z. noltii* is considered a perennial, thermophilous species, and the increasing flavonoid production during the colder seasons from September to March/April in Norway may serve as a protective function.

1. Introduction

Seagrasses are a paraphyletic group of marine hydrophilus angiosperms which evolved three to four times from land plants back to sea (Papenbrock, 2012). They play an important role in costal ecosystems and innumerous studies have been dedicated to their ecology. However, the numbers of studies exploring the secondary metabolites in *Zostera*, or other seagrass species, are limited and most of the seagrass studies measure the total amounts of compound classes and not quantities of individually characterized secondary metabolites (Cannac et al., 2006; Baby et al., 2017; Subhashini et al., 2013; Rengasamy et al., 2013a, 2013b; Zidorn, 2016; Vanitha et al., 2017). Studies on the chemical ecology of seagrasses with respect to seasonal variation of flavonoids and phenolics are also limited (Ravn et al., 1994; Rotini et al., 2013; Hernández et al., 2016; Zidorn, 2016).

Only five species of seagrasses have been found in European waters; namely *Zostera marina* Linnaeus (eelgrass), *Zostera angustifolia* (Hornemann) Reichenbach (narrow-leaved eelgrass), *Zostera noltii* Hornemann (dwarf eelgrass), *Cymodocea nodosa* Ucria (little Neptune grass) and *Posidonia oceanica* Linnaeus (Neptune grass) (Tutin et al., 1980). Three of these, *Z. marina, Z. angustifolia* and *Z. noltii* are native of Norwegian coastal waters. *Z. angustifolia* is sometimes considered a subspecies of *Z. marina* (Borum and Greve, 2004). Both taxa have 2n = 12 but they are morphological and ecological distinctive and no intermediate forms have been found in Norwegian waters (Lid and Lid, 2005). The plants we have included in this study belong to *Z. marina* sensu stricu. *Z. marina*, the most widely distributed seagrass in Norway, is most common in southern parts of Norway north to the county border between Nordland and Troms, but has also been found further north (Lid and Lid, 2005; Bekkby et al., 2011; Olsen et al., 2013). *Z. noltii* is a

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southern, thermophilous species, only known from Southeast and Southwest parts of Norway (Lid and Lid, 2005; Lundberg, 2013). *Z. noltii* is a red-listed species with status as endangered (EN).

Since Harborne and Williams in 1976 (Harborne and Williams, 1976) first reported the abundance of sulphated flavonoids in *Zostera*, and other aquatic plants, now more than 150 sulphated flavonoids have been found in nature (Correia-da-Silva et al., 2014), most of them based on flavones or flavonols. Flavonoids are in general known for their wide range of biological activities (Harborne and Williams, 2000; Yao et al., 2004; Lin and Weng, 2006; Cazarolli et al., 2008; Tapas et al., 2008; Cushnie and Lamb, 2011), such as anticoagulant (Sousa et al., 2008; Buchanan et al., 2015), anti-inflammatory, antiviral and antitumor activities (Shashank and Pandey, 2013; Teles et al., 2015). The functions of flavonoids in plants are associated with protection of the plant. Several studies have revealed that increased production of specific flavonoids in a plant can be induced by environmental stress factors, such as UV radiation, microbial and fungial attack, high or low temperatures, drought, and herbivory from insects and mammals (Jensen et al., 1998; Gould and Lister, 2006; Zidorn, 2016). It has also been suggested that flavonoids, in particular sulphated flavonoids, are involved in regulation of plant growth (Sousa et al., 2008; Buchanan et al., 2015; Teles et al., 2015). The sulphate ester bonds to flavonoids are, however, considered as relative unstable, implying that sulphated flavonoids might be degraded during extraction, purification and storage (Harborne, 1975; Barron et al., 1988). After optimization of isolation conditions the flavonoid content of *Z. marina* collected in Norwegian Sea waters were recently reported by this group using High-resolution LC-MS and NMR (Enerstvedt et al., 2016). Among the fourteen different flavones, which were identified, five were found to be sulphated and malonylated flavones were found. This was the first identification of malonylated flavones in *Zostera marina* and the second report of malonylated flavonoids isolated from marine phanerogams (Bitam et al., 2010).

In this work we characterized the qualitative and quantitative flavonoid content of *Z. noltii* found in Norwegian coastal waters for the first time. The flavonoid content of both *Z. marina* and *Z. nolti* from different sample localities were examined and seasonal variation of individual and total flavonoid concentration was investigated.

2. Material and methods

2.1. Study sites and plant collection

Samples of *Z. marina* and *Z. noltii* were collected during spring low tide by hand from eight different study sites in the southern coast of Norway (Table 1): Espegrend (A) (60°16′12.0″N, 05°13′20.3″E), Rødspollen (B) (59°36′07.8″N, 05°26′06.3″E), Strandebarm (C) (60°16′09.8″N, 06°00′56.8″E), Huglo (D) (59°51′26.9″N, 05°33′35.6″E), Gripnesvågen (E) (60°04′00.8″N, 05°39′21.6″E), Strandnesvågen (F) (58°54′26.6″N, 05°37′02.3″E), Vikerøya (G) (59°02′10.9″N, 10°08′48.9″E), Bliksekilen (H) (59°19′29.3″N, 10°29′55.5″E).

The collected plant material was washed thoroughly in fresh water and air-dried. The root was separated from the rest of the plant, and the material was cut in small pieces and stored at −20 °C, when not used. Voucher specimen of both *Z. marina* and *Z. noltii* have been deposited in the Herbarium BG at the University Museum of Bergen, Bergen. The phenolic content of fresh plant material was analyzed be HPLC prior to drying and storage. There was no evidence of degradation or alteration of the phenolic compounds during drying or storage.

Plant identification was based on microscope examination of the number of leaf nerves and formation of the leaf-tip. Flowering-stems of *Z. marina* are terminal, leaf-sheaths closed and retinacula absent. On *Z. noltii*, flowering-stems are lateral, leaf-sheaths open and retinacula present. Typically, *Z. marina* has leaves with 5–7 parallel nerves, and *Z. noltii* has leaves with 1 mid-leaf nerve. The leaf-tip of *Z. noltii* has a small notch (Lundberg, 2013).

2.2. Analytical instrumentation

Analytical HPLC: Analyses were carried out at 20 °C on an Agilent 1100 HPLC system was equipped with a HP 1050 diode array detector and a 200 × 4.6 mm inside diameter, 5 μm ODS Hypersil column (Supelco, Bellefonte, PA). Two solvents, (A) water (0.5% TFA) and (B) acetonitrile (0.5% TFA), were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by a linear gradient elution to 50% B. The flow rate was 1.0 mL/min, and aliquots of 15 μL were injected with an Agilent 1100 series microautosampler. The UV–Vis absorption spectra were recorded online during HPLC analysis over the wavelength range of 240–600 nm in steps of 2 nm.

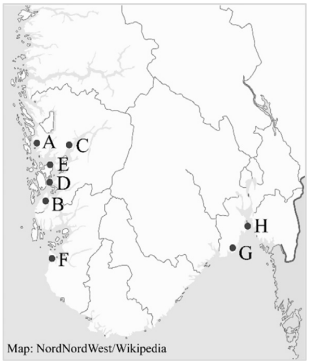
High-resolution LC-electrospray mass spectrometry (HR-LCMS) (ESI+/TOF), spectra were recorded using a JEOL AccuTOF JMS-T100LC in combination with an Agilent Technologies 1200 Series HPLC system at the following instrumental settings/conditions; Ionization mode: positive, ion source temperature = 250 °C, needle voltage = 2000 V, desolvation gas flow = 2.0 L/min, nebulizing gas flow = 1.0 L/min, orifice1 temperature = 100 °C, orifice2 voltage = 6 V, ring lens voltage = 18 V, ion guide peak voltage = 2000 V, detector voltage = 2300 V, acquisition range = 100–1000 m/z, spectral recording interval = 0.5 s, wait time = 0.03 ns and data sampling interval = 0.5 ns. The sample was solved in a mixture of water and acetonitrile with 0.1% formic or acetic acid. The elution profile for HPLC consisted of initial conditions with 90% A (water with 0.1% formic acid) and 10% B (acetonitrile with 0.1% formic acid), isocratic elution 0–2 min, followed by a linear gradient elution to 50% B (2–15 min). A 50 × 4.6 mm internal diameter, 1.8 μm Agilent Zorbax Eclipse XDB C18 column was used for separation.

2.3. Quantitative determination

Leaves of *Z. marina* and *Z. noltii* were cut into small pieces, homogenized and extracted with 50% aqueous methanol, the flavonoid content of the extract was characterized by analytical HPLC with DAD and HR- LCMS detection (Enerstvedt et al., 2016). Quantitative determination of *Z. marina*: Four replicate samples were weighed (100–200 mg) and placed into a 15 mL screw-cap glass and extracted with 7 mL of 50% aqueous methanol for 60 min at room temperature. The extract was removed and stored in a sealed glass tube. Extraction was repeated twice, and the combined extracts were transferred into a volumetric flask to determine the total volume followed by HPLC analysis. Quantitative determination of *Z. noltii*: Four replicate samples were weighed (10–50 mg) and placed into a 15 mL screw-cap glass and extracted with 3–5 mL of 50% aqueous methanol for 60 min at room temperature.

Prior to injection, the solutions were filtered through a 0.20 μm Millipore membrane filter. HPLC analysis of all the samples was carried out in triplicate and the results averaged. The quantitative amounts of the polyphenolic compounds in *Z. marina* and *Z. noltii* were determined from HPLC integration. data monitored at 360 ± 10 nm, using a calibration curve of luteolin (analytical standard, ≥ 97% (HPLC)), Sigma-Aldrich without taking into account the different molar absorption coefficients of the compounds. The results are presented as milligrams luteolin equivalents ± one

Table 1
Sample localities of *Zostera marina* and *Zostera noltii* in the southern part of Norway.



	Locality	County, municipality	Zone[a]	Depth (cm)	Plant collected
A	Espegrend	Hordaland, Bergen	sub	40-100	*Z. marina*
B	Rødspollen	Hordaland, Sveio	sub	70-140	*Z. marina*
C	Strandebarm	Hordaland, Kvam	hydro	10-50	*Z. noltii*
D	Huglo, Leira	Hordaland, Stord	hydro	10-30	*Z. noltii*
E	Gripnesvågen	Hordaland, Tysnes	sub	50-120	*Z. noltii*
F	Strandnesvågen	Rogaland, Sola	hydro	10-40	*Z. noltii*
G	Vikerøya	Vestfold, Larvik	hydro	10-60	*Z. noltii*
H	Bliksekilen	Vestfold, Tønsberg	hydro	10-60	*Z. noltii*

[a] sub = sublittoral/subtidal, hydro = hydrolittoral.

standard deviation (SD) per gram of dry weight (DW) plant material. Two sample *t*-test assuming unequal variances with a *p*-value of 0.05 was used to determine if the means of two different measurements were equal or not. Standard error bars were calculated using the STDEV. P function in excel, and represent one standard deviation (n = 4 or number of replicates).

2.4. Method validation

The established HPLC method was validated for linearity, sensitivity, precision and accuracy (Harris, 2007). Data for calibration curves, test ranges, limit of detection (LOD) and limit of quantification (LOQ) for luteolin (≥97%, Sigma-Aldrich Sigma Aldrich) is presented in Table 2. LOD and LOQ were calculated based on standard deviation of y-intercepts of the regression line (SD) and the slope (S), using the equations LOD = 3.3 × SD/S and LOQ = 10 × SD/S). The results showed good linearity, with high correlation coefficient (R^2 = 0.9989) within the test range (2.0–127.5 μg/mL). The accuracy of the HPLC method was assessed by means of spike recovery, where known amounts of the standard compound luteolin were added to extracts of *Z. marina*. Percentage recovery were calculated from the equation (Eq. (1)):

$$\%\ \text{recovery} = 100\% \times \frac{\text{Spiked sample } (\mu g) - \text{Unspiked sample}}{\text{Added } (\mu g)} \quad (1)$$

The recovery ranged from 80.21 to 82.13% (Table 3). The validation results suggested that the method developed in this paper was accurate and reliable for the quantitative analysis of the flavonoids in *Zostera marina* and *Zostera noltii*.

Table 2
Calibration curve, LOD and LOQ for Luteolin (≥97%, Sigma Aldrich).

Calibration curve[a]	R^2	Test range (μg/mL)	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
y = 69.98x−102.16	0.9989	2.0–127.5	0.19	0.56

[a] y = peak area, x = concentration (μg/mL).
[b] Limit of detection (3.3 × SD/S).
[c] Limit of quantification (10 × SD/S).

3. Results and discussion

3.1. Characterization of flavonoids in Zostera marina and Zostera noltii

In previous work (Enerstvedt et al., 2016), fourteen flavone compounds and rosmarinic acid were found in leaves of *Z. marina* from Norwegian coastal waters. In this work additional two more minor flavone sulphates, namely the 3′-sulphates of luteolin (**7**) and diosmetin (**15**) (Fig. 1), and traces of apigenin 7-sulphate (**8**) were identified, based on HPLC and HR-LCMS data (Table 4, Fig. 2). For the first time the flavonoid content of *Z. noltii* species were investigated in Norwegian coastal waters, using HPLC co-chromatography, with *Z. marina* and authentic standards, and HR-LCMS (Enerstvedt et al., 2016) (Table 4, Fig. 2). The main flavonoids found in *Z. noltii* were luteolin 7-sulphate (**4**), apigenin 7-sulphate (**8**) and diosmetin 7-sulphate (**10**), as well as rosmarinic acid (**RA**) (Fig. 1) and minor amounts of luteolin 7-glucoside (**3**), apigenin 7-glucoside (**5**) and apigenin (**13**). These results are in accordance with previous reports of *Z. noltii* (Milkova et al., 1995; Grignon-Dubois et al., 2012; Grignon-Dubois and Rezzonico, 2012). In addition the Norwegian *Z. noltii* contained minor amounts luteolin 7-(6″-malonyl)glucoside (**6**) and apigenin7-(6″-malonyl) glucoside (**11**), of which neither have been identified in *Z. noltii* before. Previously reported zosteric acid (Grignon-Dubois and Rezzonico, 2012; Laabir et al., 2013) was not found in either of the two examined Norwegian *Zostera* species. Other frequently occurring phenolic acids, such as caffeic, *p*-coumaric or chlorogenic acid were not detected.

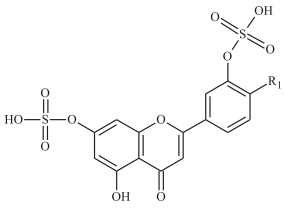
3.2. Year-to-year stability in the Zostera marina flavonoid production

Leaves of *Z. marina* were collected from the West coast locality, Espegrend (A) (Table 1) in April/May, June and in September in three subsequent years (2014–2016). The qualitative and quantitative flavonoid content was analyzed and the total flavonoid content for the different sampling times within the three years are shown in Fig. 3a. The results show a remarkable stability from year-to-year in the flavonoid production from the spring growth in April/May to the summer flush of growth in September. The predictability was both seen for the total flavonoid production as well as

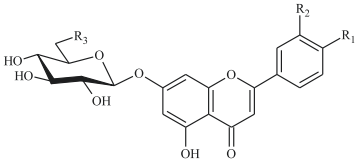
Table 3
Spike recovery study of luteolin in extracts of *Zostera marina*.

Amount of luteolin in *Z. marina* extract (μg)	Luteolin added (μg)	Luteolin in spiked sample (μg)	Recovery (%)	RSD[a] (%)
17.66	5.19	21.92	82.13	1.87
17.66	10.37	25.98	80.21	0.77
17.66	15.56	30.14	80.25	3.29

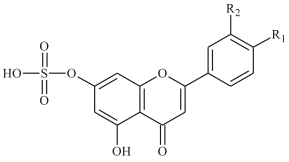
[a] Relative standard deviation (n = 3).



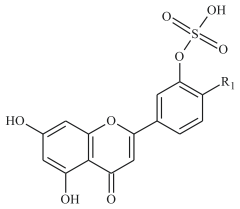
1 R_1 = OH
2 R_1 = OCH_3



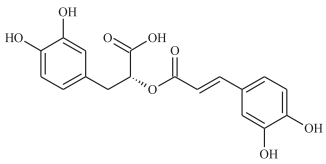
3 $R_1 = R_2 = R_3$ = OH
5 $R_1 = R_3$ = OH, R_2 = H
6 $R_1 = R_2$ = OH, R_3 = *O*-malonyl
11 R_1 = OH, R_2 = H, R_3 = *O*-malonyl
tr.2 R_1 = OH or OCH_3, R_2 = OCH_3 or OH, R_3 = *O*-malonyl



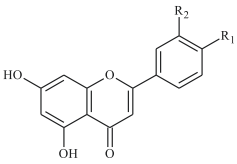
4 $R_1 = R_2$ = OH
8 R_1 = OH, R_2 = H
9 R_1 = OH, R_2 = OCH_3
10 R_1 = OCH_3, R_2 = OH



7 R_1 = OH
tr.1 R_1 = OCH_3



RA Rosmarinic acid



12 $R_1 = R_2$ = OH
13 R_1 = OH, R_2 = H
14 R_1 = OH, R_2 = OCH_3
15 R_1 = OCH_3, R_2 = OH

Fig. 1. Structures of the flavonoids and polyphenolic acid found in *Z. marina* and *Z. noltii* leaves. **1** = luteolin 7,3′-disulphate, **2** = diosmetin 7,3′-disulphate, **3** = luteolin 7-glucoside, **4** = luteolin 7-sulphate, **5** = apigenin 7-glucoside, **6** = luteolin 7-(6″-malonyl)glucoside, **7** = luteolin 3′-sulphate, **RA** = Rosmarinic acid, **8** = apigenin 7-sulphate, **9** = chrysoeriol 7-sulphate, **10** = diosmetin 7-sulphate, **11** = apigenin 7-(6″-malonyl)glucoside, **12** = luteolin, **13** = apigenin, **14** = chrysoeriol, **15** = diosmetin, **tr.1** = diosmetin 3′-sulphate, **tr.2** = diosmetin 7-(6″-malonyl)glucoside.

for the individual flavonoids (Fig. 3b). A predictability of flavonoid production from year-to-year have previously been illustrated in leaves of the terrestrial *Artemisia tridentata* subsp. wyomingensis. (Wilt and Miller, 1992). To our knowledge, the result presented in our work is the first report of year-to-year variation of individual flavonoid production in an aquatic plant.

3.3. Seasonal variation of flavonoids in Zostera marina and Zostera noltii

The variation of total flavonoid content of both *Z. marina* from Espegrend (A) and *Z. noltii* from Huglo (D) from May 2016 to March 2017 is shown in Fig. 4. The observed total flavonoid content in *Z. noltii* from another locality, Strandnesvågen (F), in June 2016 and March 2017, as well as the total flavonoid content in *Z. marina* from Rødspollen (B) in May, June and September 2016, have also been included. The difference between these two species is striking; the observed seasonal variation of *Z. noltii* from Huglo (D) showed an opposite pattern than what was observed in *Z. marina* from Espegrend (A) and Rødspollen (B). For both *Z. marina* localities, the total flavonoid content was highest in May or June, followed by a substantial drop in concentration in September. The lowest concentration in the Espegrend locality (A) was observed in February, followed by a slight increase in March. On the other hand, the flavonoid content in *Z. noltii* from Huglo (D) was lowest in June and highest in February. The two *Z. noltii* samples collected from Strandnesvågen (F) in June and March had a total flavonoid content of 27.5 and 33.2 mg/g (DW), respectively, revealing an increase of 21% in the flavonoid content from June to March, proposing a similar seasonal variation as observed in the Huglo (D) population.

The seasonal variation of individual flavonoids found in *Z. marina* at Espegrend (A) from May 2016 to March 2017 is presented in Fig. 5a. The concentration of individual flavonoids was generally highest in June, and lowest in September or February, however the variation of the disulphated flavononids (**1** and **2)** did not follow the same pattern, and their concentrations appeared

Table 4
Chromatographic and spectral (UV–vis and MS) data of the flavonoids and rosmarinic acid (**RA**) in *Zostera marina* and *Zostera noltii*.

Compound[a]	Online HPLC			LC-MS			Molecular formula
	UV_{max} (nm)	Local UV_{max} (nm)	t_R (min)	$[M+1]^+$ m/z (observed)	Fragment m/z	$[M+1]^+$ m/z (calculated)	
1	337	267	10.895	446.9725	367.0143, 287.0578	446.9692	$C_{15}H_{10}O_{12}S_2$
2	333	269	11.643	460.9869	381.0276, 301.0693	460.9848	$C_{16}H_{12}O_{12}S_2$
3	348	253, 266	12.490	449.1086	287.0562	449.1084	$C_{21}H_{20}O_{11}$
4	349	253, 266	13.966	367.0104	287.0557	367.0124	$C_{15}H_{10}O_9S$
5	337	266	14.629	433.1140	–	433.1135	$C_{21}H_{20}O_{10}$
6	338	252, 266	14.848	535.1080	–	535.1088	$C_{24}H_{22}O_{14}$
7	334	268	15.745	367.0127	287.0564	367.0124	$C_{15}H_{10}O_9S$
RA	330	290 (sh)	15.969	361.0929	163.0386	361.0923	$C_{18}H_{16}O_8$
8	338	267	16.137	351.0179	271.0602	351.0175	$C_{15}H_{10}O_8S$
9	348	252, 266	16.778	381.0283	301.0719	381.0280	$C_{16}H_{12}O_9S$
10	347	252, 266	16.977	381.0283	301.0719	381.0280	$C_{16}H_{12}O_9S$
11	337	267	17.114	519.1155	–	519.1139	$C_{24}H_{22}O_{13}$
12	346	250, 268	20.010	287.0553	–	287.0556	$C_{15}H_{10}O_6$
13[b]	332	268	22.203	271.0605	–	271.0607	$C_{15}H_{11}O_5$
14[b]	347	250, 268	23.535	301.0701	–	301.0712	$C_{16}H_{12}O_6$
15[b]	343	250, 268	23.752	301.0701	–	301.0712	$C_{16}H_{12}O_6$
tr.1[b]	–	–	–	381.0283	301.0719	381.0280	$C_{16}H_{12}O_9S$
tr. 2[b]	–	–	–	549.1242	–	549.1244	$C_{25}H_{24}O_{14}$

sh = shoulder.
[a] **1** = luteolin 7,3′-disulphate, **2** = diosmetin 7,3′-disulphate, **3** = luteolin 7-glucoside, **4** = luteolin 7-sulphate, **5** = apigenin 7-glucoside, **6** = luteolin 7-(6″-malonyl) glucoside, **7** = luteolin 3′-sulphate, RA = rosmarinic acid, **8** = apigenin 7-sulphate, **9** = chrysoeriol 7-sulphate, **10** = diosmetin 7-sulphate, **11** = apigenin 7-(6″-malonyl) glucoside, **12** = luteolin, **13** = apigenin, **14** = chrysoeriol, **15** = diosmetin, **tr.1** = diosmetin 3′-sulphate, **tr.2** = diosmetin 7-(6″-malonyl)glucoside.
[b] Only found in trace amounts in extracts.

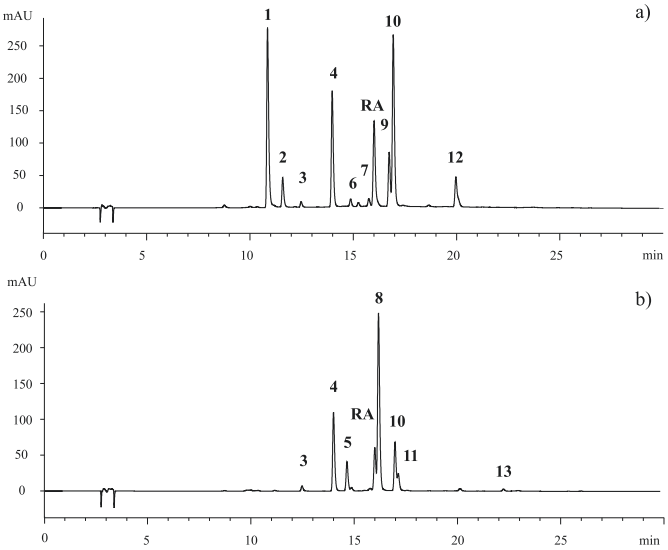


Fig. 2. HPLC profiles of extracts of *Z. marina* collected at Rødspollen (A) and *Z. noltii* collected at Vikerøya (B). See Fig. 1 for structures. The HPLC profiles are recorded at 360 ± 10 nm.

more or less unchanged throughout the year.

The seasonal variation of individual and total flavonoids found in *Z. noltii* at Huglo (D) in May, June and September in 2016, and February and March in 2017, is presented in Fig. 5b. Generally, there was a significant increase of most individual flavonoids throughout the season, with a peak concentration in either September or February, followed by a decrease in March.

3.4. Geographical variation of Zostera noltii

Leaves from six different *Z. noltii* localities (C–H), were collected in June and analyzed for their flavonoid content. As seen in Fig. 6a, there was a significant variation in both individual and total concentration of flavonoids in *Z. noltii* from the different localities. The average total flavonoid content was 27.1 ± 6.3 mg/g. Interestingly, the flavonoid content of the two populations Huglo (D) and Gripnesvågen (E), which are in close proximity to each other, differed substantially from the other populations. The population from Gripnesvågen (E), which are growing mainly sublittoral, contained the highest concentration of flavonoids of all study sites, whilst the lowest amount of flavonoids was observed in the hydrolittoral growing Huglo (C) population.

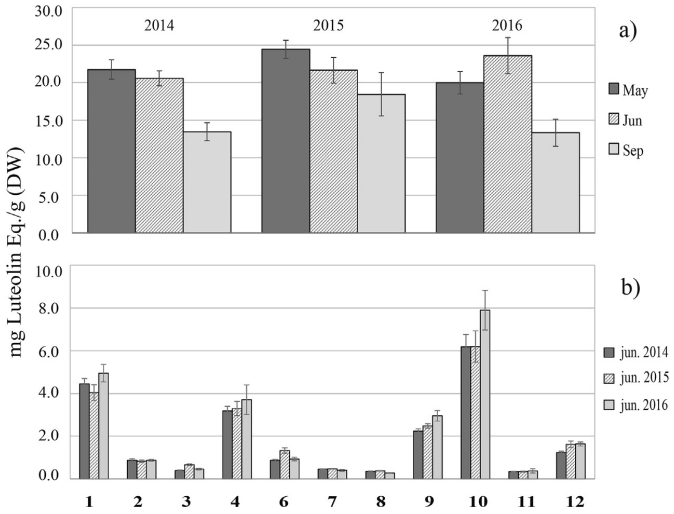


Fig. 3. a) Total flavonoid content in *Z. marina* leaves collected in April/May, June and September from Espegrend (A) in 2014, 2015 and 2016, **b)** individual flavonoid content in *Z. marina* leaves collected from Espegrend (A) in June 2014, 2015 and 2016. The flavonoid content is shown as mg Luteolin Eq./g dry weight.

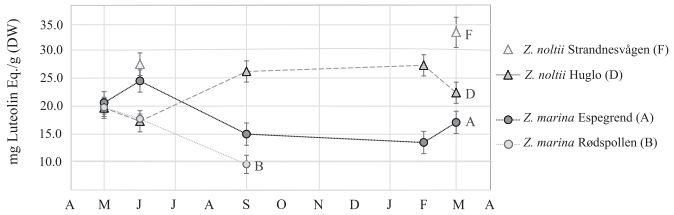


Fig. 4. Observed variation of total flavonoid content (mg Luteolin Eq./g dry weight) in *Z. marina* from Espegrend (A) and Rødspollen (B), and *Z. noltii* from Huglo (D) and Strandnesvågen (F) in the period from May 2016 to March 2017.

The two populations from Vikerøya (G) and Bliksekilen (H), both from the East coast of Norway, showed similar distribution of individual flavonoids. Apigenin 7-sulphate (**8**) was the main flavonoid, followed by luteolin 7-sulphate (**4**) and diosmetin 7-sulphate (**10**). Generally, the concentrations of apigenin based flavonoids (**5**, **8** and **11**) were significantly higher in these two populations, though the relative content of sulphated flavonoids were significantly lower compared to the populations on the West coast of Norway (Fig. 6b). Apigenin 7-sulphate (**8**) was also the main flavonoid in the Strandebarm population (C), however in the Huglo (D), Gripnesvågen (E) and Strandnesvågen (F) populations, the concentration of apigenin 7-sulphate (**8**) was considerably lower, and the main flavonoid was luteolin 7-sulphate (**4**), followed by diosmetin 7-sulphate (**10**). Grignon-Dubois and Rezzonico (2012) revealed similar geographical differences between the two *Z. noltii* populations in Cadiz and Archachon.

3.5. Quantitative amounts of rosmarinic acid

In addition to flavonoids, rosmarinic acid (RA) was one of the main compounds in *Z. marina*. As in the case of flavonoids, the concentration of RA was higher in May and June, and considerably lower in September, March and February (Fig. 5a). In *Z. marina* the amount of RA ranged from 1.0 to 3.6 mg/g (DW), whereas the RA content in *Z. noltii* was 1.3–4.5 mg/g (DW) (Fig. 6a). No significant seasonal variation of RA was observed in *Z. noltii* (Fig. 5b).

3.6. Discussion

Seagrass beds of temperate and higher latitude coastal waters show considerable seasonal changes in biomass and cover (Duarte, 1989; Vermaat and Verhagen, 1996). In the subtidal areas, which are physically and biologically relatively undisturbed, *Z. marina* forms perennial populations characterized by an asexual vegetative expansion of the rhizomes (Jacobs, 1982). To which extend *Z. marina* undergo sexual expansion in Norway is less known but low seed pollination frequency is reported at several locations (Christie et al., 2010). During autumn and winter season, most of the leaves of *Z. marina* are reduced or wither down. At the Espegrend (A) location (Table 1), we found partly green leaves also in February. The thermophilous and red-listed *Z. noltii* species, only known from Southeast and Southwest parts of Norway, is perennial and seen mainly in the hydrolittoral zone (Lundberg, 2013). *Z. noltii* also seems primarily to undergo vegetative expansion, although one can find seeds in the sediments, and flowering is observed (Lundberg, 2013). *Z. noltii* is wintergreen and in areas where the winter is mild, the biomass is seemingly unchanged as seen for our Huglo (D) location. This location (D) is also the densest growing *Z. noltii* population known in Norway, and interestingly this is where we measured the lowest flavonoid content of the examined

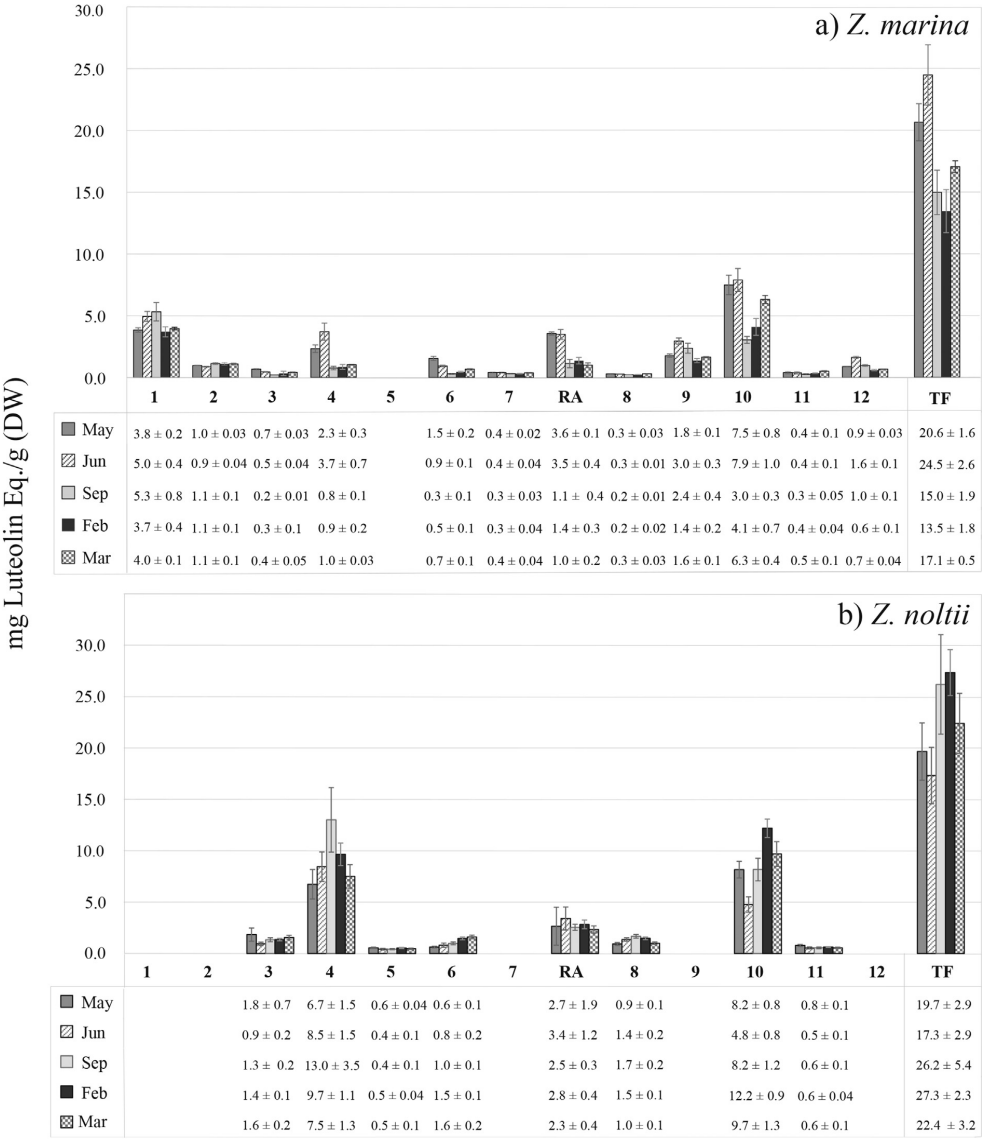
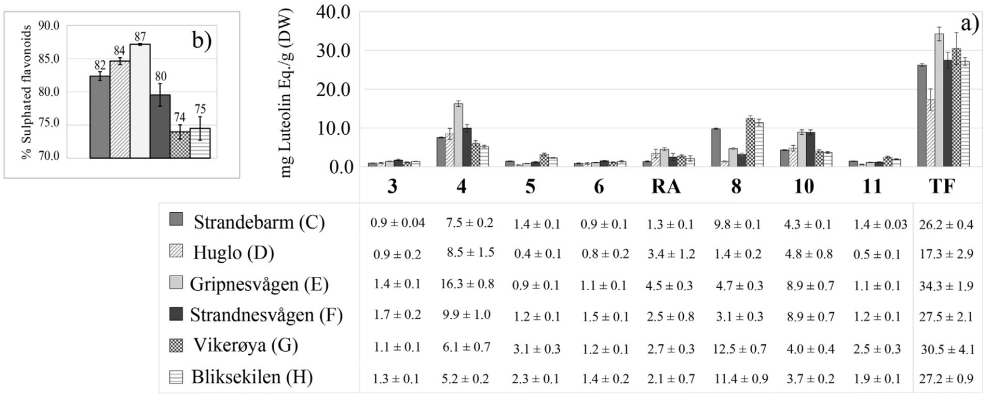


Fig. 5. Quantitative amounts (mg Luteolin Eq./g dry weight) of individual flavonoids and rosmarinic acid (RA) in leaves of **a)** *Z. marina* from Espegrend (A) and **b)** *Z. noltii* from Huglo (D) collected in spring, summer and autumn in 2016 and winter 2017. TF = total flavonoids.

Z. noltii species (Table 1, Fig. 6a). The high biomass density and lower flavonoid content may correlate to lower pressure of environmental stress factors at this site (Jacobs et al., 1981; Vergeer et al., 1995).

The variation of total flavonoid content of both *Z. marina* from Espegrend (A) and *Z. noltii* from Huglo (D) from May 2016 to March 2017 showed an opposite pattern (Fig. 4). Interestingly, the rosmarinic acid followed the flavonoid variation seen in *Z. marina* but not to the same extent in *Z. noltii*. Ravn and co-workers reported a similar seasonal pattern for caffeic and rosmarinic acid in *Z. marina* (Ravn et al., 1994), as the one observed for the flavonoids in *Z. marina* in this study; high phenolic concentrations in the younger leaves in spring, followed by lower concentrations during summer and autumn. High flavonoid concentration in late spring and early summer is strongly associated with environmental stress factors, mainly UV radiation - as seen for terrestrial plants (Chaves et al., 1997, and references therein). However, it is also likely that because the young leaves are still growing, they are consequently more vulnerable for microbial/fungal and herbivory attacks. Vergeer and Develi (1997) found that *Labyrinthula* infected



	3	4	5	6	RA	8	10	11	TF
Strandebarm (C)	0.9 ± 0.04	7.5 ± 0.2	1.4 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	9.8 ± 0.1	4.3 ± 0.1	1.4 ± 0.03	26.2 ± 0.4
Huglo (D)	0.9 ± 0.2	8.5 ± 1.5	0.4 ± 0.1	0.8 ± 0.2	3.4 ± 1.2	1.4 ± 0.2	4.8 ± 0.8	0.5 ± 0.1	17.3 ± 2.9
Gripnesvågen (E)	1.4 ± 0.1	16.3 ± 0.8	0.9 ± 0.1	1.1 ± 0.1	4.5 ± 0.3	4.7 ± 0.3	8.9 ± 0.7	1.1 ± 0.1	34.3 ± 1.9
Strandnesvågen (F)	1.7 ± 0.2	9.9 ± 1.0	1.2 ± 0.1	1.5 ± 0.1	2.5 ± 0.8	3.1 ± 0.3	8.9 ± 0.7	1.2 ± 0.1	27.5 ± 2.1
Vikerøya (G)	1.1 ± 0.1	6.1 ± 0.7	3.1 ± 0.3	1.2 ± 0.1	2.7 ± 0.3	12.5 ± 0.7	4.0 ± 0.4	2.5 ± 0.3	30.5 ± 4.1
Bliksekilen (H)	1.3 ± 0.1	5.2 ± 0.2	2.3 ± 0.1	1.4 ± 0.2	2.1 ± 0.7	11.4 ± 0.9	3.7 ± 0.2	1.9 ± 0.1	27.2 ± 0.9

Fig. 6. a) Quantitative amounts of individual flavonoids (mg Luteolin Eq./g dry weight) and rosmarinic acid in *Z. noltii* leaves collected in June 2016 from six different localities (C-H), **b)** % Sulphated of total flavonoids in *Z. noltii* leaves from the six different localities (C-H). TF = total flavonoids.

Z. marina leaves indeed had a higher phenolic content than uninfected leaves. They have also reported that lower temperatures correlated with higher content of phenolics, while lower than normal salinity was correlated to slightly higher phenolic content, but was not considered of great importance (Vergeer et al., 1995). The observed seasonal differences for the two species in our study may be related to the most obvious fact; that *Z. noltii* is a perennial, thermophilous species, increasing its flavonoid production during the colder seasons in Norway as a protective function. Other factors as reproduction strategy or increased grazing pressure by swans (*Cygnus olor*) during winter season, may affect the flavonoid production as well. Opposite of our result, Grignon-Dubois and Rezzonico (2012) report about at decreased flavonoid production in October compared to June for *Z. noltii* sampled in Spain and France. Similarly to our result though, they found a higher flavonoid production in the endangered *Z. noltii* species than in *Z. marina*. Hernández et al. (2016) revealed a seasonal variation in the seagrass *Thalassia testudinum* outside Cuba, where the total flavonoid content ranged from 9.47 mg/g (January) to 51.30 mg/g (November). However, the maximum flavonoid content in November did not correlate with temperature fluctuations, but was explained by the rainy period in October and November.

In conclusion, numbers of studies exploring the secondary metabolites in *Zostera*, or other seagrass species, are limited and most of the seagrass studies measure the total amounts of compound classes and not quantities of individually characterized secondary metabolites. In the present study, individual flavonoids of *Z. noltii* and *Z. marina* in Norwegian coastal waters were characterized and quantified. Minor amounts of luteolin 7-(6″-malonyl) glucoside (**6**) and apigenin7-(6″-malonyl)glucoside (**11**) were identified in *Z. noltii* for the first time and geographical differences were observed. The ecological significance of the structural diversification seen is unknown and warrants further investigation. The year-to-year predictability of the flavonoid production of *Z. marina* were found to be high, and *Z. marina* and *Z. noltii* seems to have different seasonal flavonoid production in Norwegian coastal waters, with the thermophilous *Z. noltii* having maximum measured production during the cold winter season.

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III

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Article

Characterization of Polyphenolic Content in the Aquatic Plants *Ruppia cirrhosa* and *Ruppia maritima* —A Source of Nutritional Natural Products

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Abstract: Herein, the polyphenolic content in extracts of *Ruppia cirrhosa* (Petagna) Grande and *Ruppia maritima* L.was fully characterized for the first time. High amounts of the main compound chicoric acid (**CA**) ($\leq$30.2 $\pm$ 4.3 mg/g) were found in both *Ruppia* species. In addition, eight flavonoids, namely the 3-*O*-glucopyranosides and 3-*O*-galactopyranosides, as well as malonylated 3-*O*-glycosides of quercetin and isorhamnetin, were isolated and identified. The antioxidant activity of *Ruppia cirrhosa* extracts and isolated compounds was investigated spectrophotometrically by a 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical scavenging assay. IC_{50} values were 31.8–175.7 µg/mL for *Ruppia cirrhosa* extracts and 12.1–88.4 µg/mL for isolated flavonoids. Both individual and total phenolic and flavonoid content were quantified in crude extracts using analytical HPLC. The relative high amount of total flavonoids ranged from 5.9 to 14.7 mg/g in both species, with concentrations of individual flavonoids ranging from 0.4 to 2.9 mg/g dry weight. The content of chicoric acid was twofold more in *Ruppia maritima* than in *Ruppia cirrhosa*. Seasonal variation of the quantitative content in *Ruppia cirrhosa* was examined. Total flavonoid content ranged from 8.4 mg/g in October to 14.7 mg/g in August, whereas the highest concentration of chicoric acid was observed in March (29.2 mg/g).

Keywords: *Ruppiaceae*; chicoric acid; flavonoids; NMR characterization; quantification; antioxidant assay

1. Introduction

The marine environment is a potential source for a wide variety of nutritional natural products. Seaweeds are used as human food or as raw materials for the production of compounds of nutritional interest [1]. On the other hand, marine angiosperms, such as seagrasses, are known for their content of secondary metabolites [2,3]; however, these are very little exploited to find commercially valuable natural products. A few seagrass species, especially of the genus *Zostera*, *Halophila*, *Posidonia*, *Thalassia* and *Syringodium*, have been investigated for their content of phenolics and flavonoids [3–13].

The widgeon grass family (*Ruppiaceae*) is a submersed aquatic angiosperm widely distributed in temperate and tropical regions all over the world. *Ruppia* species usually occur in brackish or saline waters, but can also be found in diluted fresh water or fresh water with high salinity, and only rarely under marine conditions [14–16]. In Norwegian coastal waters, two *Ruppia* species have been found, namely *Ruppia maritima* L. and *Ruppia cirrhosa* (Petagna) Grande, the latter occasionally synonymized under *R. spiralis* L. ex Dumort. Both species can be found in single populations with no other vascular plants present, and they are hardly ever found together. *R. maritima* can sometimes

be found in proximity of *Zostera noltii* populations, while *R. cirrhosa* can be found with or close to *Zostera marina* L. populations.

The number of studies investigating secondary metabolites in *Ruppia* species are limited, and a full analysis of polyphenolic content is lacking [7,10,17]. In 1973 Boutard et al. [7] analyzed and identified two flavonoids in *R. maritima* based on chrysoeriol and possibly luteolin. Harborne and Williams reported in 1976 an unidentified glycosylflavone, as well as three caffeoyl conjugates in *R. maritima*, whereas no phenolic derivatives were found in *R. cirrhosa* [10]. Haynes and Roberts indicated later the presence of flavonols in one *Ruppia* species [17], yet these results remain unpublished, and no accurate identification of the flavonols has been concluded. The previous identification work is based on TLC retention times and electrophoretic surveys [7,10].

The aim of this work was to characterize the phenolic content of *R. cirrhosa* and *R. maritima* collected from Norwegian coastal waters with the aims of finding a new source of nutritional natural products. To our knowledge, this is the first report on complete structural characterization of both flavonoids and one phenolic acid in these two species and our quantitative studies revealed high amounts of the potent chicoric acid (**CA**) [18].

2. Results and Discussion

2.1. Characterization of Polyphenolic Compounds in Ruppia cirrhosa

The HPLC profile (Figure 1) of the crude extract of *R. cirrhosa* detected at 360 ± 10 nm revealed one phenolic acid and eight flavonoids (Figure 2). After purification of the concentrated extract by Amberlite XAD-7 (Sigma-Aldrich, St. Louis, MO, USA) chromatography, the compounds were isolated by preparative HPLC and analyzed using high resolution LC-MS and 1D and 2D NMR spectroscopy. Their physiochemical and spectral data were compared to previously reported values in literature, and the compounds were identified as quercetin 3-*O*-*β*-D-galactopyranoside (**1**) [19–21], quercetin 3-*O*-*β*-D-glucopyranoside (**2**) [19,21,22], quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)galactopyranoside (**4**) [23], isorhamnetin 3-*O*-*β*-D-galactopyranoside (**5**) [24,25], isorhamnetin 3-*O*-*β*-D-glucopyranoside (**6**) [22,25,26], isorhamnetin 3-*O*-*β*-D-(6″-*O*-malonyl)galactopyranoside (**7**) [23,27], isorhamnetin 3-*O*-*β*-D-(6″-*O*-malonyl)-glucopyranoside (**8**) [27] and chicoric acid (**CA**) [28]. Quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)-glucopyranoside (**3**) was identified by comparison with an analytical standard (≥85% (HPLC), Sigma-Aldrich).

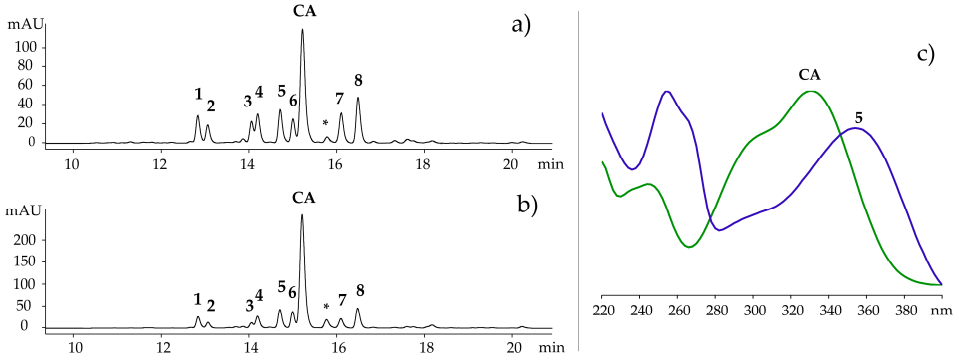
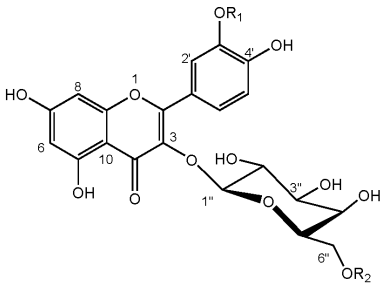
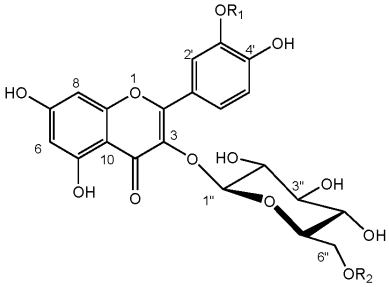


Figure 1. (**a**–**c**) HPLC chromatogram of *Ruppia cirrhosa* (**a**) and *Ruppia maritima* (**b**) recorded at 360 ± 10 nm; (**c**) UV-Vis spectrum of isorhamnetin 3-*O*-*β*-D-galactopyranoside (**5**) and chicoric acid (**CA**). See Figure 2 for structures, **1**–**8** and **CA**. * unidentified caffeoyl unit.

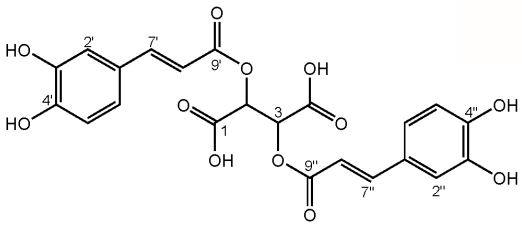
The main phenolic acid in both *Ruppia* species was chicoric acid (**CA**), which has been found previously in the seagrasses *Cymodocea nodosa* U. [29], *Syringodium filiforme* K [12], *Posidonia oceanica* L. [30–32] and

Thalassia hemprichii (Ehrenb.) Ash. [33]. This is the first time flavonoids **1**–**8** and chicoric acid have been identified in *R. cirrhosa* and *R. maritima*. The flavonoids quercetin 3-*O*-*β*-D-glucopyranoside and isorhamnetin 3-*O*-*β*-D-glucopyranoside have previously been identified in the seagrass *C. nodosa* [29]. As far as we know, this is the first report of 3-*O*-galactopyranosides and malonylated glycosides of quercetin and isorhamnetin in aquatic plants.





1	R_1 = H, R_2 = H	**2**	R_1 = H, R_2 = H
4	R_1 = H, R_2 = malonyl	**3**	R_1 = H, R_2 = malonyl
5	R_1= CH_3, R_2= H	**6**	R_1= CH_3, R_2= H
7	R_1= CH_3, R_2= malonyl	**8**	R_1= CH_3, R_2= malonyl



CA (chicoric acid)

Figure 2. Structures of the main phenolic compounds found in *Ruppia cirrhosa* and *Ruppia maritima*. **1** = quercetin 3-*O*-*β*-D-galactopyranoside, **2** = quercetin 3-*O*-*β*-D-glucopyranoisde, **3** = quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)glucopyranoside, **4** = quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)galactopyranoside, **5** = isorhamnetin 3-*O*-*β*-D-galactopyranoside, **6** = isorhamnetin 3-*O*-*β*-D-glucopyranoside, **7** = isorhamnetin 3-*O*-*β*-D-(6″-*O*-malonyl)galactopyranoside, **8** = isorhamnetin 3-*O*-*β*-D-(6″-*O*-malonyl)-glucopyranoside, **CA** = chicoric acid.

2.2. DPPH Radical Scavenging of Ruppia Polyphenols

DPPH is a stable free radical with a maximum absorbance at 517 nm (deep purple colour). When reacting with a radical scavenger it donates a hydrogen and acquires a colorless reduced form. The loss of purple colour correlates with scavenging activity of the compound, and IC_{50} values are commonly used to determine the compounds ability to scavenge radicals. The IC_{50} values of *R. cirrhosa* extracts and isolated compounds are shown in Table 1. Due to insufficient amounts of sample material, DPPH· scavenging activity of *R. maritima* was not tested. The *R. cirrhosa* extract exhibited an IC_{50} value of 152.9–175.7 μg/mL, which is considered low to moderate radical scavenging activity [34]. These results are comparable to antioxidant activities of crude extracts of the seagrasses *Halodule ovalis* (IC_{50} 130 μg/mL) [35], *Syringodium isoetifolium* (IC_{50} 96.34 μg/mL), *Enhalus acoroides* (IC_{50} 115.79 μg/mL), *Cymodocea rotundata* (IC_{50} 123.72 μg/mL) and *Thalassia hemprichii* (IC_{50} 214.68 μg/mL) [36]. However, after partition with ethyl acetate, the aqueous phase of *R. cirrhosa* exhibited very strong radical

scavenging activity, with an IC_{50} value of 31.8 ± 3.2 μg/mL. To our knowledge, this is the first reported results on DPPH· scavenging activity of *R. cirrhosa* extracts.

Table 1. IC_{50} values of extract of *Ruppia cirrhosa* and isolated compounds from *R. cirrhosa*.

Extracts and Compounds	DPPH· [1] IC_{50} (μg/mL)
R. cirrhosa crude extract (October)	175.7 ± 7.8
R. cirrhosa crude extract (August)	152.9 ± 8.1
R. cirrhosa purified extract	31.8 ± 0.7
3 + 4	12.1 ± 2.2
5 + 6	88.4 ±7.0
7 + 8	51.7 ± 6.8
CA	23.0 ± 3.2

[1] IC_{50} values calculated by linear regression of % scavenging and logarithmic concentration.

The extract from the plant material collected in October had a slightly lower scavenging activity than the *R. cirrhosa* extract from August. This may be related to the lower phenolic content found (Table 4). In addition, the percent scavenging of four crude extracts of *R. cirrhosa* with known concentrations of both flavonoids and chicoric acid was examined (Figure 3), revealing a correlation between antioxidant scavenging and concentration of total flavonoids and chicoric acid.

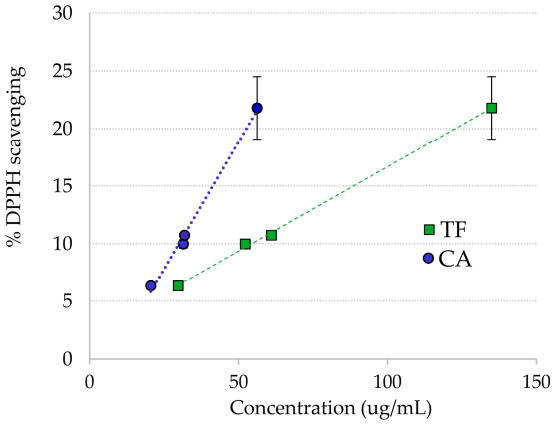


Figure 3. DPPH· radical scavenging vs. concentration of chicoric acid (**CA**) and total flavonoids (TF) in *Ruppia cirrhosa* crude extracts.

The individual flavonoids were isolated in pairs on preparative HPLC. DPPH· radical scavenging assays were performed to test the antioxidant activities of the flavonoids. The IC_{50} values of the isolated flavonoids and reference compounds are shown in Tables 1 and 2, respectively.

Purified mixture of quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)glucopyranoside (**3**) and quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)galactopyranoside (**4**) showed very strong antioxidant activity, with an IC_{50} value of 12.1 ± 3.3 μg/mL. The measured value is similar to the IC_{50} values obtained for the reference standards quercetin (5.5 ± 0.3 μg/mL), quercetin 3-*O*-*β*-D-glucopyranoside (11.0 ± 1.0 μg/mL) and rutin (13.9 ± 0.7 μg/mL), once molar mass is accounted for. Flavonoids with an isorhamnetin aglycone (compounds **5**–**8**) showed lower antioxidant activity than the quercetin-based flavonoids (**3** and **4**), explained by the number of free hydroxyl groups on the aglycone B-ring [37]. Interestingly, the malonylated isorhamnetin *O*-glycosides **7** and **8** showed much higher antioxidant activity than

the corresponding isorhamnetin *O*-glycosides **5** and **6**, with IC_{50} values of 51.7 ± 6.8 μg/mL and 88.4 ± 7.0 μg/mL, respectively.

DPPH· scavenging with chicoric acid (**CA**), isolated from *R. cirrhosa*, resulted in a higher IC_{50} value (23.0 ± 3.2 μg/mL) than the one seen for the mixture of quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)glucopyranoside (**3**) and quercetin 3-*O*-*β*-D-(6″-*O*-malonyl)galactopyranoside (**4**). Compared to the isolated isorhamnetin-based flavonoids (**5** & **6** and **7** & **8**) however, **CA** showed stronger scavenging and lower IC_{50} value. The chicoric acid (**CA**) isolated in this study had a higher IC_{50} value (23.0 ± 3.2 μg/mL) (Table 1) than the one measured for the reference compound (9.7 ± 1.7 μg/mL) (Table 2). Since DPPH is a highly concentration sensitive method, variations in IC_{50} values for the same compound is often seen [38–45]. No significant impurities were observed for the isolated sample of **CA** in the present study using HPLC and NMR for purity determination. However, water content, especially if the compound is hygroscopic, and inorganic salt content will normally not be determined by these methods [46]. Nonetheless, both the isolated **CA** and reference compound showed very strong antioxidant activity.

Table 2. IC_{50} values of reference standards.

Reference Standard	DPPH· [1] IC_{50} (μg/mL)
quercetin (≥95%)	5.5 ± 0.3
quercetin 3-*O*-*β*-D-glucopyranoside (≥90%)	11.0 ± 1.0
rutin (≥95%)	13.9 ± 0.7
Trolox (≥97%)	6.1 ± 0.4
chicoric acid (≥95%)	9.7 ± 1.7

[1] IC_{50} values calculated by linear regression of % scavenging and logarithmic concentration.

2.3. Quantitative Analysis of Polyphenolic Content in Ruppia

The quantitative content of individual flavonoids **1**–**8** and chicoric acid was characterized in three *R. cirrhosa* and two *R. maritima* populations, collected from different localities at the east and west coast of Norway (A–E) (Table 3). As seen in Figure 4a, the flavonoid content was significantly higher in *R. cirrhosa* from the Bergen location (A) compared to the other *R. cirrhosa* populations from the west coast of Norway (B and C).

Table 3. Quantitative amounts of individual flavonoids and phenolic acids in leaves of *Ruppia cirrhosa* (*R. cirr.*) and *Ruppia maritima* (*R. mar.*) collected in summer 2017 from five localities (A–E). [1,2]

Compound	*R. cirr.* (A) (mg/g)	*R. cirr.* (B) (mg/g)	*R. cirr.* (C) (mg/g)	*R. mar.* (D) (mg/g)	*R. mar.* (E) (mg/g)
CA	12.7 ± 2.5 ^{a}	11.9 ± 2.2 ^{a}	11.1 ± 1.4 ^{a}	30.2 ± 4.3 ^{b}	27.9 ± 5.1 ^{b}
1	2.2 ± 0.3 ^{d}	0.7 ± 0.1	1.1 ± 0.1 ^{g}	2.0 ± 0.5 ^{d}	1.1 ± 0.2 ^{g}
2	1.3 ± 0.2	0.5 ± 0.04 ^{e}	1.0 ± 0.1 ^{f}	1.0 ± 0.2 ^{f}	0.6 ± 0.1 ^{e}
3	0.9 ± 0.1	0.4 ± 0.04 ^{e}	0.7 ± 0.04 ^{f,g}	0.6 ± 0.1 ^{b,f}	0.6 ± 0.1 ^{b,e,g}
4	1.9 ± 0.3	0.7 ± 0.05 ^{a}	0.8 ± 0.04 ^{a}	1.5 ± 0.3 ^{b}	1.4 ± 0.2 ^{b}
5	2.9 ± 0.4	1.0 ± 0.1 ^{a}	1.0 ± 0.1 ^{a}	1.6 ± 0.3 ^{b}	2.0 ± 0.2 ^{b}
6	2.1 ± 0.2 ^{d}	0.8 ± 0.1	1.3 ± 0.1 ^{f}	1.7 ± 0.3 ^{b,d,f}	1.6 ± 0.2 ^{b}
7	1.1 ± 0.2 ^{c}	0.6 ± 0.1 ^{a,e}	0.5 ± 0.04 ^{a,f}	0.6 ± 0.07 ^{e,f}	1.1 ± 0.2 ^{c}
8	2.2 ± 0.3 ^{c}	1.1 ± 0.1	1.5 ± 0.1	1.8 ± 0.2	2.3 ± 0.3 ^{c}
sum flavonoids	14.7 ± 1.9	5.9 ± 0.5	7.9 ± 0.5	10.7 ± 1.7 ^{b}	10.7 ± 1.5 ^{b}
sum phenolics	27.4 ± 4.3	17.7 ± 2.1 ^{a}	19.0 ± 1.8 ^{a}	41.0 ± 5.7 ^{b}	38.5 ± 6.3 ^{b}

[1] Amounts are expressed in mg/g (mean value ± SD, $n = 4$) dry weigth, based on quercetin 3-*O*-β-D-glucopyranoside (flavonoids) or caffeic acid (chicoric acid) equivalents.[2] same letters (a–g) indicate where values are significantly *not* different, $p > 0.05$ with a *t* test.

No significant differences in the total flavonoid or phenolic content of the two *R. maritima* populations from the east coast were observed (D and E). However, significant differences in the distribution of the individual flavonoids were seen. The *R. maritima* samples from Tønsberg (D) showed a higher content of the quercetin *O*-glycosides **1** and **2**, whereas *R. maritima* samples from the Råde (E) location contained higher amounts of the malonylated isorhamnetin *O*-glycosides (**7**) and (**8**).

The total flavonoid content was 5.9–14.7 mg/g (DW) for *R. cirrhosa* and 10.7 mg/g (DW) for *R. maritima*, respectively (Table 3). These amounts are in the same scale as the amounts reported for common edible flavonoid sources such as *Allium* (≤5.08 mg/g DW), cranberry (2.15 mg/g DW) and dried oregano (15.46 mg/g DW) [47–50]. In marine European seagrass species as *Zostera marina* and *Zostera noltii* flavonoid amounts in the range of 13.5–24.5 mg/g (DW) and 3.38–34.3 mg/g (DW) have been found, respectively [9,51].

The concentrations of chicoric acid (**CA**) were significantly higher in *R. maritima* (30.2 and 27.9 mg/g) than in *R. cirrhosa* (11.1–12.7 mg/g). It seems natural to conclude that *R. maritima* generally have a higher production of **CA** although, although it should be taken into consideration that the *R. maritima* samples were collected from a different part of Norway. Differences in chicoric acid accumulation may be a function of nutritional and/or environmental stress, but there is a need for more research on how chicoric acid accumulation in plants is regulated [18]. In leaves of *Cymodocea nodosa* and *Syringodium filiforme*, the amounts of chicoric acid have been reported to range from 8.13–27.4 mg/g and 0.94–5.26 mg/g, respectively [12,29]. Chicoric acid has also been found in *Posidionia oceania* from the Mediterranean Sea, however, the quantitative content varied greatly. The maximum content of chicoric acid was 0.1386 mg/g in young leaves of *P. oceanica* collected in the Aegean sea outside Turkey, whereas both detrital and fresh leaves of *P. oceanica* from four different localities in the western part of the Mediterranean sea were found to contain up to 12.78 mg/g chicoric acid [31,32]. The high level of **CA** (≤30.2 ± 4.3 mg) found in this study is comparable to the content of **CA** in the known source *Echinacea purpura* [52–54], proposing *Ruppia* to be a new and valuable source of chicoric acid (**CA**). Chicoric acid is high value-added on the nutraceutical market, due to its possible health benefits and its relative rare occurrence in the plant kingdom [12,18].

Fluctuations in natural product concentrations should be taken into consideration before scheduling harvest dates or planning herbal product manufacturing [18]. In order to get an impression of the seasonal fluctuations of phenolics in *Ruppia*, the total flavonoid and **CA** content in *R. cirrhosa* collected from the Bergen location (A) in October, March and August were analyzed (Table 4, Figure 4b). During the winter season (December-February) the biomass on the examined locality was scarce.

Table 4. Quantitative amounts of individual flavonoids and chicoric acid in leaves of *Ruppia cirrhosa* collected in October 2016, March 2017 and August 2017. [1,2]

Compound	16 October (mg/g)	17 March (mg/g)	17 August(mg/g)
CA	10.6 ± 2.5 [a]	29.2 ± 6.3	12.7 ± 2.5 [a]
1	0.8 ± 0.1	2.2 ± 0.4 [b]	2.2 ± 0.3 [b]
2	0.6 ± 0.1	0.8 ± 0.2	1.3 ± 0.2
3	0.7 ± 0.1 [a]	0.9 ± 0.2 [a]	0.9 ± 0.1 [a]
4	1.1 ± 0.2	2.5 ± 0.6 [b]	1.9 ± 0.3 [b]
5	1.1 ± 0.2	1.5 ± 0.3	2.9 ± 0.4
6	0.8 ± 0.1 [a]	0.7 ± 0.1 [a]	2.1 ± 0.2
7	1.2 ± 0.2 [a]	1.1 ± 0.2 [a]	1.1 ± 0.2 [a]
8	2.0 ± 0.3	1.4 ± 0.3	2.2 ± 0.3
sum flavonoids	8.4 ± 1.1 [a]	11.1 ± 2.4 [a]	14.7 ± 1.9
sum phenolics	19.0 ± 3.0	40.3 ± 8.7	27.4 ± 4.3

[1] Amounts are expressed in mg/g (mean value ± SD, $n = 4$) dry weight, based on quercetin 3-*O*-β-D-glucopyranoside (flavonoids) or caffeic acid (chicoric acid) equivalents. [2] same letters (a,b) indicate where values are significantly *not* different, $p > 0.05$ with a t test.

The concentration of flavonoids in *R. cirrhosa* was significantly higher in August (14.7 ± 1.9 mg) compared to October (8.4 ± 1.1 mg) and March (11.1± 2.4 mg). The concentration of **CA** in *R. cirrhosa* measured in March (29.2 ± 6.3 mg) was over twice the amounts found in August (12.7 ± 2.5) and October (10.6 ± 2.5). The observed seasonal variation of flavonoids and phenolic acid indicates a similar pattern as we have previously seen in *Zostera marina* [51], with higher concentrations in spring and summer. These trends are associated with environmental stress factors, mainly UV radiation—as seen for terrestrial plants [55,56]. It is also likely that because the young leaves are still growing, they are consequently more vulnerable for microbial/fungal and herbivory attacks, which will result in an increased production of phenolics [57]. Yet, to achieve more accurate and reliable data on the seasonal variation in relation to environmental factors, a more comprehensive study of the content of both flavonoids and chicoric acid in *R. maritima* and *R. cirrhosa* is needed.

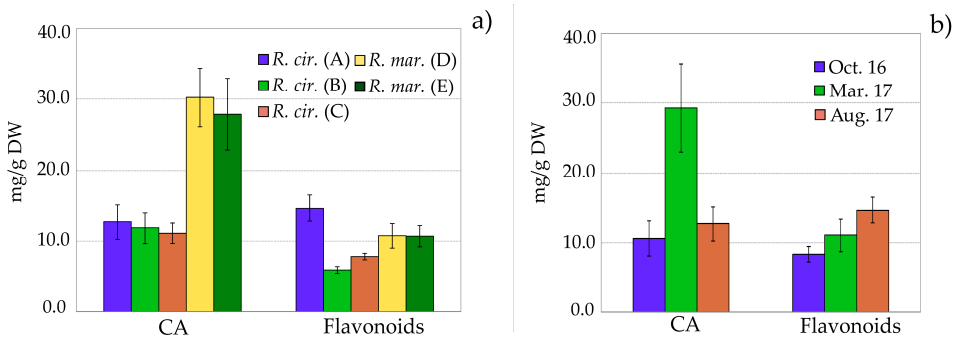


Figure 4. (**a**) Flavonoid and chicoric acid (**CA**) content in leaves of *Ruppia cirrhosa* (*R. cirr.*) and *Ruppia maritima* (*R. mar.*) collected from different localities; (**b**) Flavonoid and chicoric acid (**CA**) content in leaves of *Ruppia cirrhosa* collected in October 2016, March 2017 and August 2017. Amounts are expressed in mg/g (mean value ± SD, n = 4) dry weight, based on quercetin 3-*O*-*β*-D-glucopyranoside (flavonoids) or caffeic acid (**CA**) equivalents.

3. Experimental

3.1. General Instrumentation

3.1.1. Analytical HPLC

Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a HP 1050 diode array detector and a 200 × 4.6 mm inside diameter, 5 µm ODS Hypersil column (Supelco, Bellefonte, PA, USA). Two solvents, (A) water (0.5% TFA) and (B) acetonitrile (0.5% TFA), were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by a linear gradient elution to 50% B. The flow rate was 1.0 mL/min, and aliquots of 15 µL were injected with an Agilent 1100 series microautosampler. The UV-Vis absorption spectra were recorded online during HPLC analysis over the wavelength range of 240–600 nm in steps of 2 nm.

3.1.2. Preparative HPLC

The preparative HPLC system used a Gilson 321 pump (Gilson S. A., Villiers-le-Bel, France), equipped with an Ultimate 3000 variable wavelength detector (Dionex, Thermo Fisher Scientific, Sunnyvale, CA, USA), a 25 × 2.12 cm (10 µm) UniverSil C18 column (Fortis Technologies Ltd., Neston, UK), and the solvents (A) water (0.1% formic acid) and (B) acetonitrile (0.1% formic acid). The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by isocratic elution for the next 5 min, and the subsequent linear gradient conditions: 5–18 min (to 16% B), 18–22 min (to 18% B), 22–26 min (to 23% B), 26–31 min (to 28% B), and 31–32 min (to 40% B), with isocratic

elution at 32–40 min (40% B) and a final linear gradient elution at 40–43 (to 10% B). The flow rate was 15 mL/min, and aliquots of 800 µL were injected.

3.1.3. LC–MS

High-resolution LC-electrospray mass spectrometry (HR-LCMS) (ESI+/TOF), spectra were recorded using a AccuTOF JMS-T100LC (JEOL, Peabody, USA) in combination with an Agilent Technologies 1200 Series HPLC system at the following instrumental settings/conditions; Ionization mode: positive, ion source temperature = 250 °C, needle voltage = 2000 V, desolvation gas flow = 2.0 L/min, nebulizing gas flow = 1.0 L/min, orifice1 temperature = 100 °C, orifice2 voltage = 6 V, ring lens voltage = 18 V, ion guide peak voltage = 2000 V, detector voltage = 2300 V, acquisition range = 100–1000 m/z, spectral recording interval = 0.5 s, wait time = 0.03 ns and data sampling interval = 0.5 ns. The sample was dissolved in a mixture of water and acetonitrile with 0.1% formic acid. The elution profile for HPLC consisted of initial conditions with 90% A (water with 0.1% formic acid) and 10% B (acetonitrile with 0.1% formic acid), isocratic elution 0–2 min, followed by a linear gradient elution to 50% B (2–15 min). A 50 × 4.6 mm internal diameter, 1.8 µm Agilent Zorbax Eclipse XDB C18 column was used for separation.

3.1.4. NMR-Spectroscopy

One-dimensional ^{1}H and ^{13}C distortionless enhancement by polarization transfer (DEPT-135), two-dimensional heteronuclear single quantum coherence (^{1}H-^{13}C HSQC), heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC), heteronuclear 2 bond correlation (^{1}H-^{13}C H2BC), double quantum filtered correlation (^{1}H-^{1}H DQF COSY), heteronuclear single quantum coherence-total correlation spectroscopy (^{1}H-^{13}C HSQC-TOCSY), homonuclear *J*-resolved (^{1}H *J*-RES) and total correlation spectroscopy (^{1}H-^{1}H TOCSY) experiments were obtained on a Bruker 850 MHz instrument (Bruker BioSpin, Zürich, Switzerland) equipped with a cryogenic probe. The spectral widths were 10–15 ppm and 165–220 ppm for the ^{1}H and ^{13}C-dimensions, respectively. The number of collected data points was 2048 for ^{1}H-dimension in most 2D experiment (4096 in HMBC), and 256 in the ^{13}C dimension. The 2D experiments HMBC, HSQC and H2BC were acquired with non-uniform sampling (NUS = 20–50%). The coupling constants were 145 Hz for $^{1}J_{CH}$, 8 Hz for long range couplings (HMBC) and 120–160 Hz for $^{2}J_{CH}$ (H2BC). Recycle delay was 2 s in all experiments. Sample temperatures were stabilized at 298 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (d_6-DMSO or d_4-MeOD) were used as secondary references (δ 39.5/2.5 and 49.1/3.31 from TMS, respectively).

3.2. Plant Material and Study Sites

Samples of *R. cirrhosa* and *R. maritima* were collected during spring low tide by hand from five different study sites in the southern coast of Norway: Bergen, Røytepøyla (A) (60°15′34.5″ N, 05°15′57.9″ E), Etne, Gjersvik, (B) (59°38′41.5″ N, 05°55′18.8″ E), Tysvær, Hadleholmen (C) (59°23′44.1″ N, 05°28′29.6″ E), Tønsberg, Bliksekilen (D) 59°19′25.7″ N, 10°29′58.2″ E) and Råde, Skjeløy (E) (59°17′00.4″ N, 10°44′33.5″ E). Voucher specimen of *Ruppia cirrhosa* and *Ruppia maritima* have been deposited in the Herbarium BG (Voucher BG/S 164805 and 53439) at the University Museum of Bergen, Bergen. Plant identification was based on plant morphology and habitat ecology. Leaves of both species are brown-greenish, narrowly linear, sheathering at the base, and fine teethed at the apex. Sheaths of *R. maritima* are slightly inflated; sheaths of *R. cirrhosa* are typically conspicuously inflated. Flowers of both species are hermaphroditic and small, in two-flowered, pedunculate spikes. Perianth is absent. Peduncles in *R. cirrhosa* are 8–15 cm long, sometimes longer, and spirally coiled when fruits are mature. Peduncles in *R. maritima* are shorter; 4–6 cm long, often somewhat recurved in fruit but never spirally coiled. *R. cirrhosa* is typically 30–50 cm long, whereas *R. maritima* often is 10–15 cm long, sometimes up to 30 cm long. *R. maritima* is found mostly in the hydrolittoral zone, sometimes down to the upper part of the sublittoral zone, growing at ±0.5 m deep, whereas *R. cirrhosa* occurs in the

sublittoral zone and is permanently submerged at depths of 0.5–1.5 m. Both species are found on soft substrata, such as mud and silt. *R. maritima* is also found on fine sand.

3.3. Extraction, Purification and Identification

The collected plant material was washed thoroughly in fresh water and air-dried. The root was separated from the rest of the plant, and the material was cut in small pieces and stored at −20 °C, when not used. Air-dried leaves of *R. cirrhosa* were extracted with 50% aqueous methanol (HPLC) for 24 h at room temperature. The extraction was repeated 4 times. The combined extracts were filtered through glass wool, and the volume was further reduced using a rotavapor. The concentrated aqueous extract was partitioned against ethyl acetate three times. The content of both the ethyl acetate and water phase was examined on HPLC. About a third of the aqueous extract was applied to an Amberlite XAD-7 column (5 × 20 cm), and eluted with distilled water until no colour was observed, then methanol was applied. Collected fractions were analyzed on analytical HPLC and concentrated using a rotavapor. The semi-purified plant extract was submitted to preparative HPLC to obtain purified compounds. The physiochemical and spectral data of the flavonoids and chicoric acid were as follows: *Quercetin 3-O-β-*D*-galactopyranoside* (**1**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 353, 256, 264 (sh); HRLC-MS *m*/*z* 465.1015 $[M + H]^+$, ^{1}H-NMR (d_6-DMSO, 850.13 MHz) δ (ppm), aglycone; 7.66 (1H, dd, *J* = 8.6, 2.3 Hz, H-6′), 7.53 (1H, d, *J* = 2.3 Hz, H-2′), 6.82 (1H, d, *J* = 8.4 Hz, H-5′), 6.41 (1H, d, *J* = 1.9 Hz, H-8), 6.20 (1H, d, *J* = 1.9 Hz, H-6), sugar; 5.37 (1H, d, *J* = 7.7 Hz, H-1″), 3.56 (1H, m, H-2″), 3.37 (1H, dd, *J* = 9.6, 3.6 Hz, H-3″), 3.65 (1H, m, H-4″), 3.33 (1H, m, H-5″), 3.29 (1H, dd, *J* = 10.8, 5.8 Hz, H-6a″), 3.46 (1H, dd, *J* =10.8, 6.2 Hz, H-6b″). ^{13}C-NMR (d_6-DMSO 213.765 MHz) δ (ppm): aglycone; 156.2 (C-2), 133.5 (C-3), 177.5 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 121.1 (C-1′), 116.0 (C-2′), 144.9 (C-3′), 148.5 (C-4′), 115.2 (C-5′), 122.0 (C-6′), sugar; 101.8 (C-1″), 71.2 (C-2″), 73.2 (C-3″), 67.9 (C-4″), 75.9 (C-5″), 60.1 (C-6″). The structure was confirmed by comparison with literature data [19–21].

*Quercetin 3-O-β-*D*-glucopyranoside* (**2**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 352, 256, 263 (sh); HRLC-MS *m*/*z* 465.0999 $[M + H]^+$, ^{1}H-NMR (d_6-DMSO, 850.13 MHz) δ (ppm), 7.66 (1H, dd, *J* = 8.6, 2.3 Hz, H-6′), 7.53 (1H, d, *J* = 2.3 Hz, H-2′), 6.82 (1H, d, *J* = 8.6 Hz, H-5′), 6.41 (1H, d, *J* = 1.9 Hz, H-8), 6.20 (1H, d, *J* = 1.9 Hz, H-6), sugar; 5.46 (1H, d, *J* = 7.4 Hz, H-1″), 3.24 (1H, t like, *J* = 8.4 Hz H-2″), 3.22 (1H, t, *J* = 8.5 Hz, H-3″), 3.09 (1H, d, *J* = 5.7 Hz, H-4″), 3.08 (1H, m, H-5″), 3.32 (1H, td, *J* = 12.0, 6.0, 2.1 Hz, H-6a″), 3.58 (1H, d, *J* = 12.0 Hz, H-6b″). ^{13}C-NMR (d_6-DMSO 213.765 MHz) δ (ppm): aglycone; 156.2 (C-2), 133.3 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.5 (C-8), 156.3 (C-9), 104.0 (C-10), 121.2 (C-1′), 116.2 (C-2′), 144.8 (C-3′), 148.5 (C-4′), 115.2 (C-5′), 122.0 (C-6′), sugar; 100.8 (C-1″), 74.1 (C-2″), 76.5 (C-3″), 69.9 (C-4″), 77.6 (C-5″), 60.9 (C-6″). The structure was confirmed by comparison with literature data [19,21,22,58].

*Quercetin 3-O-β-*D*-(6″-*O*-malonyl)galactopyranoside* (**4**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 354, 256, 264 (sh); HRLC-MS *m*/*z* 551.1062 $[M + H]^+$, ^{1}H-NMR (d_6-DMSO, 850.13 MHz) δ (ppm): aglycone; 7.67 (1H, dd, *J* = 8.3, 2.3 Hz, H-6′), 7.52 (1H, d, *J* = 2.2 Hz, H-2′), 6.81 (1H, d, *J* = 8.6 Hz, H-5′), 6.40 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6), sugar; 5.37 (1H, d, *J* = 8.1 Hz, H-1″), 3.57 (1H, m, H-2″), 3.36 (1H, dd, *J* = 8.9, 3.7 Hz, H-3″), 3.65 (1H, m, H-4″), 3.61 (1H, td, *J* = 6.2, 1.7 Hz, H-5″), 4.00 (1H, dd, *J* = 12.0, 5.8Hz, H-6a″), 4.20 (1H, dd, *J* =12.1, 2.3 Hz, H-6b″), acyl; 3.11 (2H, d, *J* = 16.0 Hz, H-2‴). ^{13}C-NMR (d_6-DMSO 213.765 MHz) δ (ppm): aglycone; 156.2 (C-2), 133.4 (C-3), 177.1 (C-4), 161.2 (C-5), 98.6 (C-6), 164.1 (C-7), 93.4 (C-8), 156.3 (C-9), 103.8 (C-10), 121.5 (C-1′), 116.2 (C-2′), 144.7 (C-3′), 148.4 (C-4′), 115.1 (C-5′), 121.9 (C-6′), sugar; 101.7 (C-1″), 71.0 (C-2″), 73.1 (C-3″), 67.9 (C-4″), 72.4 (C-5″), 63.5 (C-6″), malonyl; 166.5 (C-1‴), 41.0 (C-2‴), 167.7 (C-3‴). The structure was confirmed by comparison with literature data [23].

*Isorhamnetin 3-O-β-*D*-galactopyranoside* (**5**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 351, 254, 266 (sh); HRLC-MS *m*/*z* 479.1208 $[M + H]^+$, ^{1}H-NMR (d_4-MeOD, 850.13 MHz) δ (ppm): aglycone;

7.59 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), 8.03 (1H, d, *J* = 2.0 Hz, H-2′), 6.90 (1H, d, *J* = 8.4 Hz, H-5′), 6.41 (1H, d, *J* = 1.9 Hz, H-8), 6.21 (1H, d, *J* = 1.9 Hz, H-6), 3.96 (3H, s, OCH_3), sugar; 5.34 (1H, d, *J* = 7.4 Hz, H-1″), 3.82 (1H, dd, *J* = 9.6, 7.8 Hz, H-2″), 3.56 (1H, dd, *J* = 9.1, 2.8 Hz, H-3″), 3.84 (1H, dd, *J* = 3.2, 0.9 Hz, H-4″), 3.48 (1H, t, *J* = 8.5 Hz, H-5″), 3.47 (1H, td, *J* = 11.7, 5.7, 1.7 Hz, H-6a″), 3.65 (1H, dd, *J* = 11.8, 6.1 Hz, H-6b″). ^{13}C-NMR (d_4-MeOD, 213.765 MHz) *δ* (ppm): aglycone; 158.8 (C-2), 135.6 (C-3), 179.6 (C-4), 163.3 (C-5), 100.0 (C-6), 166.1 (C-7), 94.9 (C-8), 158.6 (C-9), 105.9 (C-10), 123.2 (C-1′), 114.7 (C-2′), 148.6 (C-3′), 151.0 (C-4′), 116.1 (C-5′), 123.8 (C-6′), 57.0 (OCH_3), sugar; 104.5 (C-1″), 73.3 (C-2″), 75.2 (C-3″), 70.2 (C-4″), 77.4 (C-5″), 62.3 (C-6″). The structure was confirmed by comparison with literature data [24,25].

*Isorhamnetin 3-O-β-*D*-glucopyranoside* (**6**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 354, 254, 266 (sh); HRLC-MS *m*/*z* 479.1212 $[M + H]^+$, ^{1}H-NMR (d_4-MeOD, 850.13 MHz) *δ* (ppm): aglycone; 7.58 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 7.93 (1H, d, *J* = 2.0 Hz, H-2′), 6.91 (1H, d, *J* = 8.3 Hz, H-5′), 6.41 (1H, d, *J* = 1.9 Hz, H-8), 6.21 (1H, d, *J* = 1.9 Hz, H-6), 3.95 (3H, s, OCH_3), sugar; 5.41 (1H, d, *J* = 7.8 Hz, H-1″), 3.46 (1H, t like, *J* = 8.6 Hz, H-2″), 3.45 (1H, dd, *J* = 9.4, 8.2 Hz, H-3″), 3.30 (1H, m, *J* = 9.4 Hz, H-4″), 3.24 (1H, m, H-5″), 3.57 (1H, dd, *J* = 11.9, 5.8 Hz, H-6a″), 3.73 (1H, dd, *J* = 12.0, 2.5 Hz, H-6b″). ^{13}C-NMR (d_4-MeOD, 213.765 MHz) *δ* (ppm): aglycone; 158.8 (C-2), 135.5 (C-3), 179.6 (C-4), 163.3 (C-5), 100.0 (C-6), 166.1 (C-7), 94.9 (C-8), 158.6 (C-9), 105.9 (C-10), 123.2 (C-1′), 114.6 (C-2′), 148.6 (C-3′), 151.0 (C-4′), 116.1 (C-5′), 122.5 (C-6′), 57.0 (OCH_3), sugar; 103.7 (C-1″), 76.1 (C-2″), 78.2 (C-3″), 71.6 (C-4″), 78.7 (C-5″), 62.7 (C-6″). The structure was confirmed by comparison with literature data [22,25,26].

*Isorhamnetin 3-O-β-*D*-(6″-O-malonyl)galactopyranoside* (**7**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 350, 254, 266 (sh); HRLC-MS *m*/*z* 565.1216 $[M + H]^+$, ^{1}H-NMR (d_4-MeOD, 850.13 MHz) *δ* (ppm): aglycone; 7.62 (1H, dd, *J* = 8.3, 2.1 Hz, H-6′), 7.90 (1H, d, *J* = 2.0 Hz, H-2′), 6.92 (1H, d, *J* = 8.4 Hz, H-5′), 6.44 (1H, d, *J* = 2.1 Hz, H-8), 6.23 (1H, d, *J* = 2.1 Hz, H-6), 3.97 (3H, s, OCH_3), sugar; 5.21 (1H, d, *J* = 7.6 Hz, H-1″), 3.81 (1H, m, H-2″), 3.58 (1H, t, *J* = 9.7 Hz, H-3″), 3.89 (1H, d, *J* = 4.3 Hz, H-4″), 3.86 (1H, t, *J* = 9.1 Hz, H-5″), 4.29 (1H, dd, *J* = 11.4, 4.4 Hz, H-6a″), 4.49 (1H, dd, *J* = 11.6, 8.4 Hz, H-6b″). ^{13}C- NMR (d_4-MeOD, 213.765 MHz) *δ* (ppm): aglycone; 157.6 (C-2), 134.0 (C-3), 178.0 (C-4), 161.7 (C-5), 98.5 (C-6), 164.6 (C-7), 93.6 (C-8), 157.0 (C-9), 104.3 (C-10), 121.5 (C-1′), 113.0 (C-2′), 146.9 (C-3′), 149.5 (C-4′), 114.7 (C-5′), 122.4 (C-6′), 55.4 (OCH_3), sugar; 103.3 (C-1″), 71.4 (C-2″), 73.4 (C-3″), 69.0 (C-4″), 73.4 (C-5″), 63.1 (C-6″), acyl; 166.3 (C-1‴). The structure was confirmed by comparison with literature data [23,27].

*Isorhamnetin 3-O-β-*D*-(6″-O-malonyl)glucopyranoside* (**8**): Yellow amorphous powder (MeOH); UV/Vis λ_{max} nm 355, 254, 266 (sh); HRLC-MS *m*/*z* 565.1208 $[M + H]^+$, ^{1}H-NMR (d_4-MeOD, 850.13 MHz) *δ* (ppm): aglycone; 7.61 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), 7.88 (1H, d, *J* = 2.1 Hz, H-2′), 6.91 (1H, d, *J* = 8.4 Hz, H-5′), 6.44 (1H, d, *J* = 2.1 Hz, H-8), 6.22 (1H, d, *J* = 2.1 Hz, H-6), 3.95 (3H, s, OCH_3), sugar; 5.22 (1H, d, *J* = 7.6 Hz, H-1″), 3.40 (1H, m, *J* = 8.6 H-2″), 3.43 (1H, t, *J* = 8.7 Hz, H-3″), 3.35 (1H, t, *J* = 9.7 Hz, H-4″), 3.47 (1H, t, *J* = 8.5 Hz, H-5″), 4.19 (1H, dd, *J* = 12.0, 5.6 Hz, H-6a″), 4.23 (1H, dd, *J* = 12.0, 2.3 Hz, H-6b″). ^{13}C-NMR (d_4-MeOD, 213.765 MHz) *δ* (ppm): aglycone; 157.7 (C-2), 135.6 (C-3), 173.9 (C-4), 161.7 (C-5), 98.5 (C-6), 164.6 (C-7), 93.6 (C-8), 157.1 (C-9), 104.3 (C-10), 121.6 (C-1′), 113.0 (C-2′), 147.0 (C-3′), 149.6 (C-4′), 114.6 (C-5′), 122.7 (C-6′), 55.4 (OCH_3), sugar; 103.1 (C-1″), 74.2 (C-2″), 76.5 (C-3″), 69.9 (C-4″), 74.4 (C-5″), 63.4 (C-6″), acyl; 169.0 (C-1‴). The structure was confirmed by comparison with literature data [27].

2,3-O-Dicaffeoyltartaric acid (**CA**): White amorphous powder (MeOH); UV/Vis λ_{max} nm 331, 302 (sh), 245; HRLC-MS *m*/*z* 497.0681 $[M + Na]^+$, ^{1}H-NMR (d_6-DMSO, 850.13 MHz) *δ* (ppm): 5.68 (2H, s, H-2, H-3), 7.10 (2H, d, *J* = 2.1 Hz, H-2′, H-2″), 6.78 (2H, d, *J* = 8.1 Hz, H-5′, H-5″), 7.08 (2H, dd, *J* = 8.2, 2.1 Hz, H-6′, H-6″), 7.56 (1H, d, *J* = 15.8 Hz, H-7′, H-7″), 6.36 (1H, d, *J* = 15.8 Hz, H-8′, H-8″). ^{13}C-NMR (d_6-DMSO, 213.765 MHz) *δ* (ppm): 167.6 (C-1, C-4), 70.7 (C-2, C-3), 125.2 (C-1′, C-1″), 115.3 (C-2′, C-2″), 145.6 (C-3′, C-3″), 148.9 (C-4′, C-4″), 115.8 (C-5′, C-5″), 121.7 (C-6′, C-6″), 147.0 (C-7′, C-7″), 112.3 (C-8′, C-8″), 165.5 (C-9′, C-9″). The structure was confirmed by comparison with literature data [28].

3.4. Quantitative Determination

Leaves of *R. cirrhosa* and *R. maritima* were cut into small pieces and extracted with 50% aqueous methanol, the flavonoid content of the extract was characterized by analytical HPLC with DAD and HR–LCMS. Quantitative determination: 10–40 mg of dried plant material was weighed and extracted with 3–5 mL of 50% aqueous methanol for 2 hours at room temperature. Four replicate samples were made. Prior to injection, the solutions were filtered through a 0.45 µm Millipore membrane filter. HPLC calibration curves of quercetin 3-*O*-*β*-D-glucopyranoside (≥90% (HPLC), Sigma-Aldrich, Sigma-Aldric, St. Louis, MO, USA) and caffeic acid (≥98% (HPLC), Sigma-Aldrich) were used to determine the quantitative amounts of flavonoids and phenolic compounds, respectively. The results are presented as milligrams quercetin 3-*O*-*β*-D-glucopyranoside *or* caffeic acid equivalents ± one standard deviation (SD) per gram of dry weight (DW) plant material. Two sample t-test assuming unequal variances with a *p*-value of 0.05 was used to determine if the means of two different measurements were equal or not. Standard error bars were calculated using the STDEV. P function in excel, and represent one standard deviation (n = 4 or number of replicates).

3.5. Method Validation

The established HPLC method was validated for linearity, sensitivity, precision and accuracy, as previously described [51]. LOD and LOQ were calculated based on standard deviation of y-intercepts of the regression line (SD) and the slope (S), using the equations LOD = 3.3 × SD/S and LOQ = 10 × SD/S. Recovery study was performed in triplicate by adding known amounts of quercetin 3-*O*-*β*-D-glucopyranoside to crude extracts of *R. cirrhosa*. Data for calibration curves, test ranges, limit of detection (LOD) and limit of quantification (LOQ) for quercetin 3-*O*-*β*-D-glucopyranoside (90%, Sigma-Aldrich Sigma) and caffeic acid (Sigma-Aldrich) are presented in Table 5. The recovery was ranging from 93.3% to 94.8% for quercetin 3-*O*-*β*-D-glucopyranoside with a mean of 94.0 ± 2.0% (Table 5).

Table 5. Calibration curve, LOD and LOQ for quercetin 3-*O*-*β*-D-glucopyranoside (≥90%, Sigma Aldrich) and caffeic acid (≥98%, Sigma-Aldrich).

	Calibration Curve (µg/mL)	R^2	Test Range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Spike Recovery %
quercetin 3-*O*-*β*-D-glucopyranoside	$y = 36.56x - 11.8$	0.9998	2.5–80	2.0	6.0	94.0 ± 2.0
caffeic acid	$y = 102.8x + 12.8$	0.9994	10–80	1.1	3.3	

3.6. DPPH Radical Scavenging

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH·) was used for determination of free radical-scavenging activity of *R. cirrhosa* extracts and isolated mixtures of flavonoids (purity ≥ 75% (HPLC)). Different sample concentrations of the extracts were prepared, and 0.05 mL of each sample was added to a 2.95 mL methanolic solution of DPPH· (45 µg/mL). A UV-1800 UV spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) was used for the antioxidant assays. The UV/Vis absorbance at 517 nm was measured every 30 s for 5 min. The experiment was repeated three times, and the results are presented as mean ± standard deviation (n = 3). Trolox (97%, Sigma-Aldrich), chicoric acid (≥95% (HPLC), Sigma-Aldrich), quercetin (≥95% (HPLC), Sigma-Aldrich), quercetin 3-*O*-*β*-D-glucopyranoside (≥90% (HPLC), Sigma-Aldrich) and rutin (≥95% (HPLC), Sigma-Aldrich) were used as standard controls. Percent radical-scavenging was calculated as $100 \times (A_{start} - A_{end})/(A_{start})$, where A_{start} is the absorbance before addition of the sample, and A_{end} is the absorbance value after 5 min of reaction time. Percent scavenging IC_{50} values were calculated from a linear regression plot of percent scavenging (%) against logarithmic concentration of the test compound [59]. IC_{50} values denote the concentration of sample which is required to scavenge 50% of DPPH· free radicals.

4. Conclusions

In this study, the polyphenolic content of *Ruppia cirrhosa* and *Ruppia marittima* was characterized for the first time using NMR-spectroscopy, HRLC-MS and HPLC-UV. Both *Ruppia* species contained high amounts of chicoric acid (10.6–30.2 mg/g DW), followed by relatively high amounts of flavonoid glycosides (5.9–14.7 mg/g DW). The eight flavonoids identified were based on quercetin and isorhamnetin with 3-*O*-galactopyranosides or 3-*O*-glucopyranosides, four of these were malonylated. This is the first report of 3-*O*-galactopyranosides and malonylated flavonoids of quercetin and isorhamnetin isolated from aquatic plants. The seasonal variations of flavonoids and phenolics were examined by analyzing *R. cirrhosa* samples in October, March and August. Highest flavonoid content was found in August, whereas the highest concentration of chicoric acid was observed in March.

Extracts of *R. cirrhosa* showed low to moderate DPPH· antioxidant activity, however, partially purified extract and isolated compounds showed strong to very strong antioxidant activities, with IC_{50} values ranging from 12.1 to 88.4 µg/mL.

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Conflicts of Interest: The authors declare no conflict of interest.

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