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Synthetic analogues of marine natural flavonoids as antifouling agents: synthesis and biological evaluation

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Work developed under the scientific supervision of Professor Honorina Maria de Matos Cidade and Professor Marta Ramos Pinto Correia da Silva Carvalho Guerra

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- **Poster communications**

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> "If your dreams do not scare you, they are not big enough."
>
> Ellen Johnson Sirleaf

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Abstract

Both macro and microorganisms are part of the great community responsible for the process known as biofouling, which occurs when certain marine species attach to natural or artificial underwater surfaces and accumulate. Several economic and environment problems are associated and investments have been made in order to discover new ecofriendly antifouling agents.

Marine environment has been considered as a potential resource for environmentally friendly natural antifouling agents in recent years. In fact, many structurally diverse marine natural products (MNP) have been reported as antifouling compounds, including the glycosylated flavone sulfate thalassiolin A.

Inspired by the potential of this marine flavone as non-toxic antifouling agents, three new thalassiolin A structure related flavone glycosides were obtained by total synthesis. Firstly, flavone derivatives **LuMe**, **TriCe**, **LuNi**, and **LuCl** were synthesized by Mentzer synthesis, and afterward **LuMe** and **TriCe** were used as building blocks for the synthesis of one *O*-acetyl glycoside derivative (**LuMeGluAc**) and two flavone *O*-acetyl glycoside sulfates (**LuMeTGluS** and **TriCeTGluS**), possessing a 1,2,3-triazole ring as a linker between the flavone nucleus and the sulfated glycosyl moiety. Flavone **LuMeGluAc** was synthesized by the reaction of **LuMe** with acetobromo-*α*-D-glucose in the presence of silver carbonate. Flavones **LuMeTGluS** and **TriCeTGluS** were obtained by Click chemistry, involving the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) of previously formed 7-*O*-propargyl derivatives **LuMeProp** and **TriCeProp** and 2,3,4-tetra-*O*-acetyl-β-glucopyranosyl azide, followed by deprotection of the glycosidic moiety with sodium methoxide and the sulfation with sulfur trioxide adduct.

The antifouling potential of all synthesized compounds, including the synthetic intermediates, as well as a small library of structure related flavonoids previously synthesized by LQOF/CIIMAR research group, were assessed using the anti-settlement activity of mussel (*Mytilus galloprovincialis*) plantigrade post-larvae. Among the tested compounds, six compounds have been identified as promising antifouling agents, including **TriCe** and **LuNi**. For the most promising compounds, *in silico* studies concerning the prediction of biodegradability and bioaccumulation in aquatic environment were conducted. The results of biological activity assays combined with these *in silico* studies suggest the potential of **TriCe** as a new non-toxic antifouling agent.

Keywords: flavones, antifouling agents, sulfated glycosides.

Resumo

Os macro- e os microorganismos constituem uma parte da grandiosa comunidade responsável pelo processo de bioincrustação, que ocorre quando certas espécies marinhas se ligam, e se acumulam, a superfícies subaquáticas naturais ou artificiais. Este processo está associado a vários problemas económicos e ambientais que levaram a que investimentos fossem feitos de forma a descobrir novos agentes anti-incrustantes sem toxicidade para o ambiente marinho.

Nos últimos anos, o ambiente marinho foi considerado como um potencial recurso para a obtenção de agentes anti-incrustantes naturais amigos do ambiente. Efetivamente, muitos produtos naturais marinhos estruturalmente diversificados foram descritos como compostos anti-incrustantes, incluindo a flavona glicosilada sulfatada, talassiolina A.

Inspirado pelo potencial não-tóxico anti-incrustante desta flavona marinha, três novas flavonas glicosiladas, estruturalmente relacionadas com a talassiolina A, foram obtidas por síntese total. Inicialmente, as flavonas **LuMe**, **TriCe**, **LuNi** e **LuCl** foram sintetizados pela síntese de Mentzer e, posteriormente, a **LuMe** e **TriCe** foram utilizadas como blocos construtores para a síntese do derivado *O*-acetil-glicosídico (**LuMeGluAc**) e dos derivados *O*-acetil-glicosídicos sulfatados de flavona (**LuMeTGluS** e **TriCeTGluS**), possuindo um anel 1,2,3-triazol como ligação entre o núcleo da flavona e a porção glicosídica sulfatada. A flavona **LuMeGluAc** foi sintetizada pela reação da **LuMe** com acetobromo-*α*-D-glucose, na presença de carbonato de prata. As flavonas **LuMeTGluS** e **TriCeTGluS** foram obtidas por química "click", através da cicloadição dos derivados de 7-*O*-propargilo **LuMeProp** e **TriCeProp**, previamente sintetizados, e da 2,3,4-tetra-*O*-acetil-*β*-glucopiranosilazida, seguida de reação de desprotecção com metóxido de sódio, e de reação de sulfatação, com aducto de trióxido de enxofre.

Posteriormente, foi avaliado o potencial anti-incrustante de todos os compostos sintetizados, incluindo os intermediários sintéticos, bem como de uma pequena biblioteca de flavonóides estruturalmente relacionados, previamente sintetizados pelo grupo de pesquisa LQOF/CIIMAR, utilizando a atividade de anti-deposição de larvas plantigráficas de mexilhão (*Mytilus galloprovincialis*). Entre os compostos testados, seis compostos foram identificados como promissores agentes anti-incrustantes, incluindo as flavonas **TriCe** e **LuNi**. Para os compostos mais promissores, foram efetuados estudos *in silico* tendo em vista prever a biodegradação e bioacumulação no ambiente marinho. Os resultados dos ensaios biológicos combinados com os dos estudos *in silico* sugerem o potencial da flavona **TriCe** como novo agente anti-incrustante não-tóxico.

Palavras-chave: flavonas, agentes anti-incrustantes, glicosídeos sulfatados.

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Abbreviations and Symbols

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
brs	broad singlet
$CDCl_3$	Deuterated chloroform
CIIMAR	Interdisciplinary centre of marine and environmental research
d	doublet
dd	double doublet
CuAAC	Copper(I)-catalyzed azide alkyne cycloaddition
DMSO	Dimethylsulphoxide
EC_{50}	Half maximal effective concentration
EPA	Environmental protection agency
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
IR	Infrared spectroscopy
LC_{50}	Half maximal lethal concentration
log k_{ow}	Octanol-water partition coefficient
LQOF	Laboratório de Química Orgânica e Farmacêutica
LuCl	5,7-Dihydroxy-4'-chloroflavone
LuMe	Luteolin 3',4'-dimethyl ether
LuMeGluAc	7-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)luteolin 3',4'-dimethyl ether
LuMeProp	7-(prop-2-yn-yloxy)luteolin 3',4'-dimethyl ether
LuMeTGluAc	7-((2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1H-1,2,3-triazole-4-yl)methoxy)luteolin 3',4'-dimethyl ether
LuMeTGluOH	7-((*β*-D-glucopyranosyl)-1H-1,2,3-triazole-4-yl)methoxy)luteolin 3',4'-dimethyl ether

LuMeTGluS	7-((2,3,4,6-tetrasulfate-*β*-D-glucopyranosyl)-1H-1,2,3-triazole-4-yl)methoxy)luteolin 3',4'-dimethyl ether
LuNi	5,7-Dihydroxy-4'-nitroflavone
m	multiplet
MAOS	Microwave-assisted organic synthesis
MIC	Minimum inhibitory concentration
MNP	Marine natural products
Mp	Melting point
MW	Microwave
NMR	Nuclear magnetic resonance
PTC	Phase-transfer-catalyzed
q	Quadruplet
r.t.	room temperature
s	singlet
SAR	Structure-activity relationship
t	triplet
TBAB	Tetrabutylammonium bromide
TLC	Thin layer chromatography
TriCe	Tricetin trimethyl ether
TriCeProp	7-(prop-2-yn-yloxy)tricetin trimethyl ether
TriCeTGluAc	7-((2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1H-1,2,3-triazole-4-yl)methoxy)tricetin trimethyl ether
TriCeTGluOH	7-((*β*-D-glucopyranosyl)-1H-1,2,3-triazole-4-yl)methoxy)tricetin trimethyl ether
TriCeTGluS	7-((2,3,4,6-tetrasulfate-*β*-D-glucopyranosyl)-1H-1,2,3-triazole-4-yl)methoxy)tricetin trimethyl ether
UV	Ultraviolet
δc	Carbon chemical shift
δ_H	Proton chemical shift
υ	Wavenumver

Outline of the dissertation

This dissertation is organized into six chapters:

CHAPTER I - Introduction

The first chapter deals with the theoretical background that supports the developed work and so is sub-divided into five subchapters. Initially, the theme relating to the marine world is introduced and then focuses on the marine natural flavonoids, namely their structure and biological activity, as well as the methods that can be used for the synthesis of some marine flavonoids. Finally, an overview of the approach taken, as well as the main objectives of the present dissertation are described.

CHAPTER II - Results and Discussion

This chapter focus on the results and discussion of the developed research work. In several subchapters are discussed the results obtained concerning the synthesis, biological activity, and biodegradability and bioaccumulation in *in silico* studies.

CHAPTER III - Conclusions and Perspectives

The third chapter focus on the main conclusions and future work.

CHAPTER IV - Experimental Procedures

Herein the procedures adopted for the synthesis, evaluation of biological activity, and *in silico* biodegradability and bioaccumulation studies are described.

CHAPTER V - References

The fifth chapter presents a list of the references used in this dissertation. The references followed the American Chemical Society style guide. The main bibliographic research motors were ISI Web of Knowledge, from Thomson Reuters, Scopus, and Google Scholar.

APPENDIX 1

Appendix 1 is an article submitted to publication that reviews the natural distribution, structure, and biological activities of marine flavonoids.

CHAPTER I

Introduction

1. Marine world as a source of bioactive compounds

As more than 70% of the world's surface is covered by oceans, marine organisms offer a rich and unlimited resource of structurally diverse bioactive compounds which make up approximately one half of the total global biodiversity.[1-2] However, it is one of the most underutilized biological resources, containing a vast array of organisms with unique biological systems and characteristics.[2]

Ecological pressures on marine organisms are very different from terrestrial environment [1] and due to the wide range of environments, marine organisms have developed unique properties and bioactive compounds that, in some cases, are unparalleled by their terrestrial counterparts. This characteristic allows them to survive under conditions with varying degrees of salinity, pressure, temperature, and illumination.[2]

Marine organisms are divided in four categories: microorganisms (microalgae, bacteria, and fungus), plants (seagrass, mangrove, halophyte, angiosperm, brown algae, red algae, and green algae), invertebrates (mollusc, coral, crustaceans, sponges, sea cucumbers, ascidians, etc.), and vertebrates, from simple to complex in accordance with evolution. These organisms produce unique secondary metabolites to fight against situations like competition for space, deterrence of predation, and the ability to reproduce.[3] In recent years, general aspects of the chemical structures, physical and biochemical properties, and biotechnological applications of bioactive substances derived from marine organisms, plants or animals have been exhaustively studied.[4]

MNP have attracted the attention of biologists and chemists all over the world. Marine flora and fauna represent a virtually untapped resource for identifying and extracting biologically active substances with potential to act as nutritional supplements, pharmaceuticals, cosmetics, agrochemicals, molecular probes, enzymes, fine chemicals, and antifouling agents.[5]

Biomolecules derived from marine organisms are useful to the food industry in a number of applications, including efficient food production under unique conditions such as low temperature or high pressure; providing added nutritional benefits to foods; and using as "natural" pigments, preservatives, flavors, colorants, stabilizers, and gelling agents.[2, 5] Marine bioactive compounds can be used in the nutraceutical and pharmaceutical industries, as actives in pharmaceuticals, nutraceuticals, dietary supplements, and prebiotics due of their antioxidant, antitumor, antimicrobial, antiviral, antiaging, antithrombotic, anticoagulant, anti-inflammatory, antihypertensive, antidiabetic, and cardioprotective activities.[5]

MNP have also been considered as one of the most promising sources of antifouling compounds in recent years. In fact, many of marine macroorganisms are able to stay free from biofouling and their secondary metabolites are believed to be chemical defensive substances. These MNP are easily biodegradable and leave no residue in the environment, thus have been considered as a potential resource for environmentally friendly natural antifouling agents.[6-7] In the past decades, many MNP with a variety of structural types have been reported as non-toxic antifouling compounds.[8-9]

2. Flavonoids

Flavonoids are secondary metabolites with low molecular weight and polyphenolic nature, which have a considerable scientific and therapeutic interest.[10-11]

Flavonoids have a common structure of diphenylpropanes, C6-C3-C6. They possess a fifteen-carbon skeleton consisting of two benzene rings (rings A and B) that are linked by a three carbon unity, which may or may not form a third ring (ring C) (**Figure 1**).[11-12]

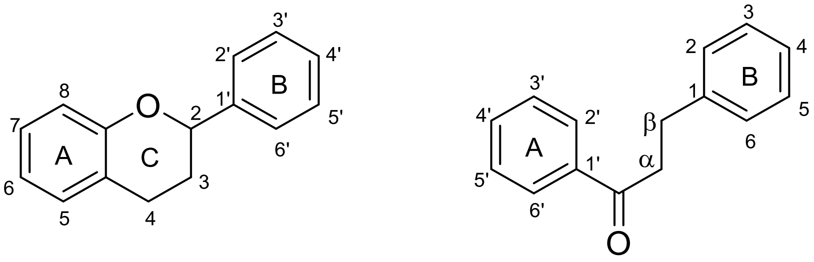
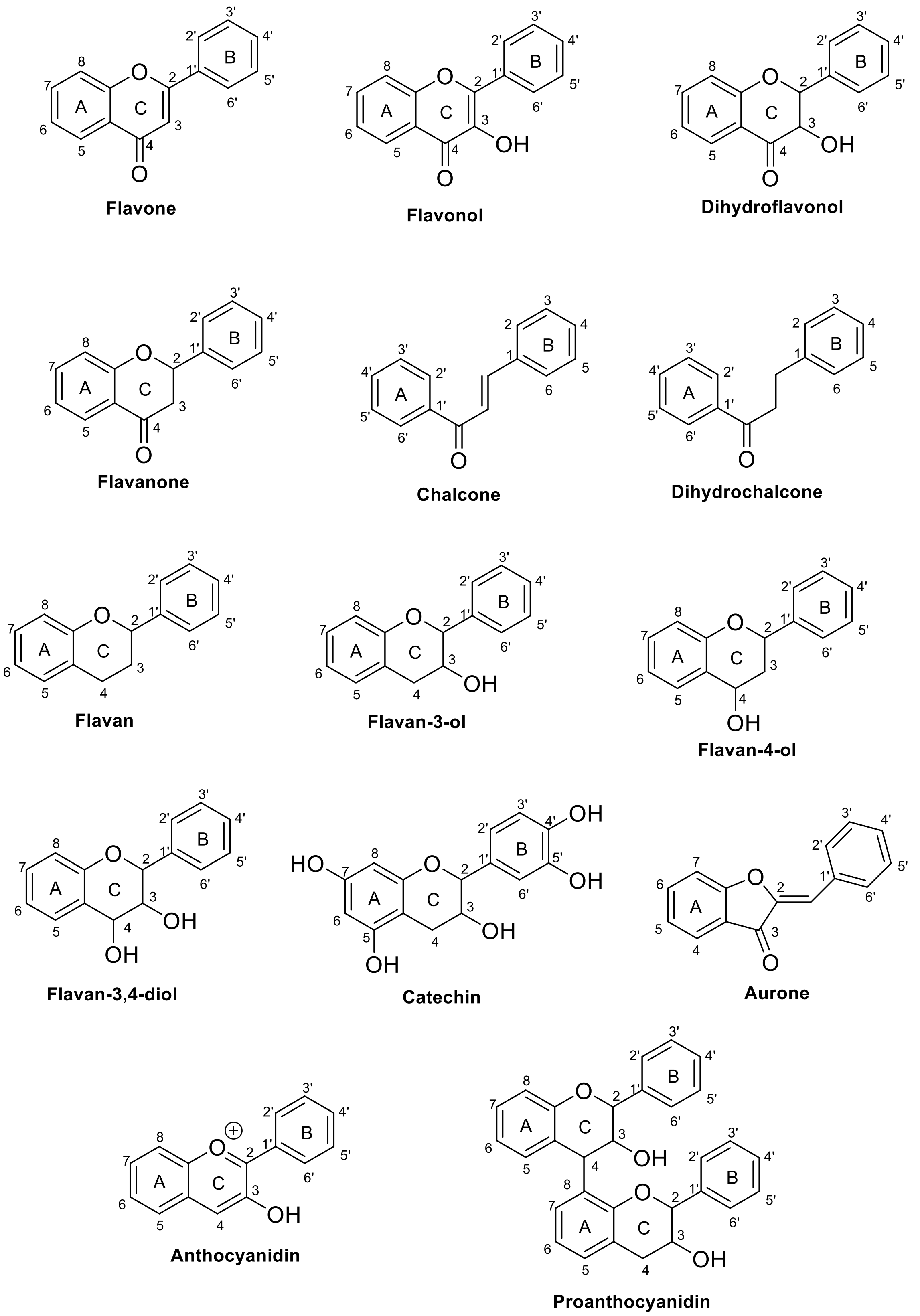


Figure 1 - General skeleton of flavonoids.

Variations in oxidation level of the carbon atoms of the C ring and type of substituents are the key feature determining the flavonoid family. They can be classified into several subclasses, namely flavones, isoflavones, flavonols, dihydroflavonols, flavanones, chalcones, dihydrochalcones, flavans, flavan-3-ols, catechins, aurones, anthocyanins, and proanthocyanidins (**Figure 2**).[11, 13]



Flavone

Flavonol

Dihydroflavonol

Flavanone

Chalcone

Dihydrochalcone

Flavan

Flavan-3-ol

Flavan-4-ol

Flavan-3,4-diol

Catechin

Aurone

Anthocyanidin

Proanthocyanidin

Figure 2 – Several subclasses of flavonoids.

In addition, the basic structure of flavonoids allows a multitude of substitution patterns in the A and B rings within each class of flavonoids, including hydroxyl and methoxy groups, sugars, sulfates, and glucuronides, among other groups.[14] The *C*-hydroxylation and *C*-methoxylation, as well as *C*- and *O*-methylation, and glycosylation can be selectively introduced into different positions of the molecule, and the diverse positions, number, and combinations of these substituents contribute to the enormous variability of the flavonoid scaffold.[15]

Biosynthetically, A ring is derived from the "acetate pathway", by head-to-tail condensation of three "acetate units", forming a resorcinol or phloroglucinol moiety. While, B ring is derived from an unsaturated phenolic acid resulting from the "shikimate pathway". The condensation of the resorcinol or phloroglucinol moiety with the carboxyl group of an unsaturated phenolic acid results in the generation of chalcones, which are considered the precursors of other flavonoids. Chalcones can after undergo cyclization to flavanone derivatives, a reversible process catalysed by specific chalcones-flavanone isomerases (**Figure 3**).[15-16] The presence or absence of a hydroxyl-containing side chain on the phenolic acid results in generation of the two main subgroups of flavonoids: the 3-desoxyflavonoids (chalcones, flavanones, and flavones) and the 3-hydroxyflavonoids (flavonols, anthocyanidins, and catechins).[16]

From the chalcone-flavanone system, several pathways guide to the other flavonoid subgroups. The reaction controlled by four sequentially arranged genes transforms the C9 precursor unit *via* chalcones, flavanones, and flavanonols into 3-flavanols and anthocyanidins. Another pathway from chalcones to flavonoids, bypassing the flavanones, is provided by reduction of chalcones and cyclization to 3-flavones, which may be converted to anthocyanins, flavandiols, and catechins. On the other hand, chalcones are also precursors of flavones either by direct conversion or by mediation of flavanones which are dehydrogenated to flavones (**Figure 3**).[15]

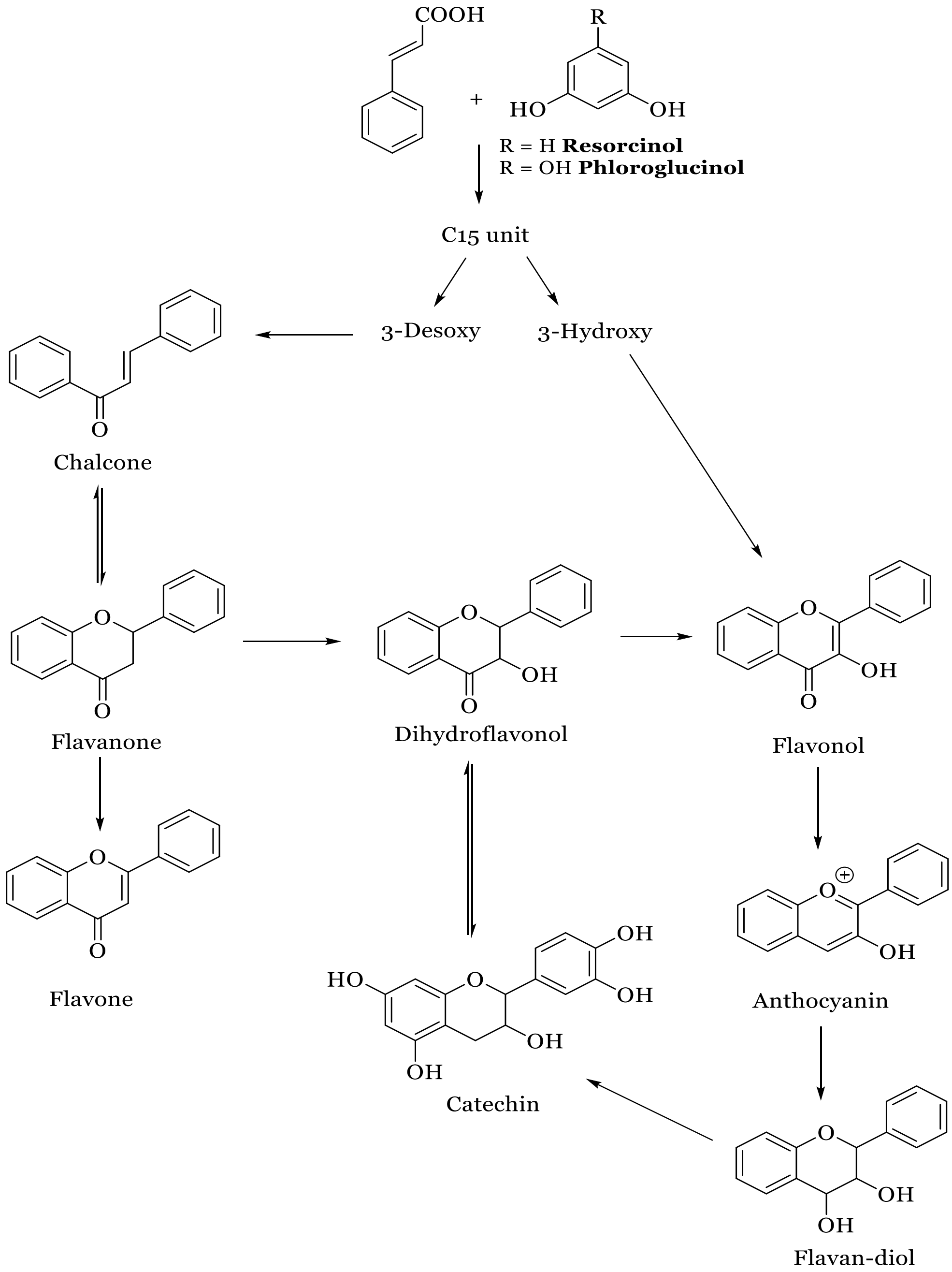


Figure 3 - Biosynthesis of flavonoids.

3. Flavonoids isolated from marine sources with antifouling activity

Over the past 50 years, the reports about marine flavonoids isolation have been increasing, especially flavones and flavonols. Flavonoids isolated from marine sources, as well as their biological activities are described in **Appendix I**.

Some marine natural flavonoids have been attracting the scientific community because of their unique substitution pattern and promising biological activities. In fact, several marine flavonoids have been reported has having not only pharmacological activities (antioxidant, antimicrobial, antitumor, anticoagulant, and antidiabetic activities), but also antifouling activity, demonstrating their potential as lead compounds for pharmaceutical and chemical industries.

The formation of biofouling involves the attachment of a range of micro- and macroorganisms in natural and artificial underwater surfaces, and causes serious technical and operational challenges for the maritime industries.[8] Considering this, efforts have been on the development of non-toxic and environmentally friendly antifouling agents, which inhibit the biofilm formation and protect against marine biofouling organisms, such as pathogen bacteria, and other intruders. These agents should be capable to inhibit the settlement of selected biofouling species by acting in more specific signalling targets, somehow related with settlement processes, instead of inducing general toxicity.[8]

In the past decades, some flavonoids with different substitution pattern were reported as potential antifouling agents (**Figure 4**). Jensen *et al.*[17] demonstrated that the crude extract of healthy *Thalassia testudinum* leaf tissues inhibited the growth of the co-occurring thraustochytrid *Schizochytrium aggregatum* and prevented the attachment of *S. aggregatum* motile zoospores to an extract-impregnated substrate.[17] A bioassay-guided chemical fractionation of this extract resulted in the isolation of thalassiolin A (luteolin-7-*O*-*β*-D-glucopyranosyl-2”-sulfate), a new sulfated flavone glycoside, which displayed antibiotic activity against *S. aggregatum* with a half maximal inhibitory concentration (IC_{50}) of 270 μg.mL^{-1}, and reduced the attachment of *S. aggregatum* motile zoospores to an extract-impregnated substrate. These results suggest the ecological role of thalassiolin A in chemical defense against fouling microorganisms.[17]

More recently, two thalassiolin A structure related flavones, luteolin and luteolin-4’-glucuronide, isolated from the seagrass *Enhalus acoroides*, also revealed antibacterial activity against bacterial strains isolated from marine biofilms (*Loktanella hongkongensis, Rhodovulum* sp, and *Vibrio halioticoli*).[18] Luteolin inhibited the growth of *L. hongkongensis* with a minimum inhibitory concentration (MIC) of 50 μg.mL^{-1} and had slight inhibition towards *Rhodovulum* sp., while luteolin 4’-glucuronide inhibited the growth of the bacteria *L. hongkongensis* and *V. halioticoli* at high concentrations (200 μg.mL^{-1}).[18] In addition to the antibacterial activity, luteolin 4’-glucuronide inhibited larval settlement of *Bugula neritina* effectively, with an half maximal effective concentration (EC_{50}) value of 0.52 μg.mL^{-1}, with low toxicity.[18] Overall results suggest that luteolin can be a lead compound to the discovery of new antifouling agents.

In 2013, Bao *et al.*[19] studied the ethyl acetate (EtOAc) extract of a culture broth of the marine-derived fungus *Penicillium* sp., isolated from South China Sea gorgonian coral *Dichotella gemmacea* and concluded that this extract exhibited significant antifouling activity against *Balanus amphitrite* larvae settlement. Further investigation on the chemical constituents of the extract led to the isolation of a new flavone, 6,8,5',6'-tetrahydroxy-3'-methylflavone, with potential antifouling activity. This flavone strongly inhibited the *B. amphitrite* larvae settlement with EC_{50} value of 6.71 $\mu g.mL^{-1}$, showing no toxicity toward larvae at concentrations of 3.13–100 $\mu g.mL^{-1}$, which indicated that the LC_{50}/EC_{50} values was > 14.[19]

Kong *et al.*[20] studied the antibacterial activity of flavonoids isolated from the leaves of the halophyte *Apocynum venetum* against marine biofilm-derived bacteria *Bacillus thuringiensis*, *Pseudoalteromonas elyakovii*, and *Pseudomonas aeruginosa*. 8-Methylretusin showed weak antibacterial activity towards *B. thuringiensis* and *P. aeruginosa* (inhibition zone diameter of 9.4±0.6 and 10.5±0.9 mm, respectively), but no activity against *P. elyakovii.* Quercetin and (-)-catechin displayed weak antibacterial activity against all tested bacteria (inhibition zone diameter between of 9.2±0.5 and 10.9±0.7 mm, respectively), while kaempferol exhibited moderate antibacterial activities towards all tested bacteria (*B. thuringiensis*: inhibition zone diameter of 17.4±0.9 mm, *P. elyakovii*: inhibition zone diameter of 9.5±0.6 mm, *P. aeruginosa:* 15.8±0.8 mm).[20]

Developing antifoulants from marine sources demands high compound amount, which often cannot be met by re-isolation from the respective marine source. In this direction, the search for alternatives for resupply, namely biotechnology approaches and/or total organic synthesis worth to be explore in the future.

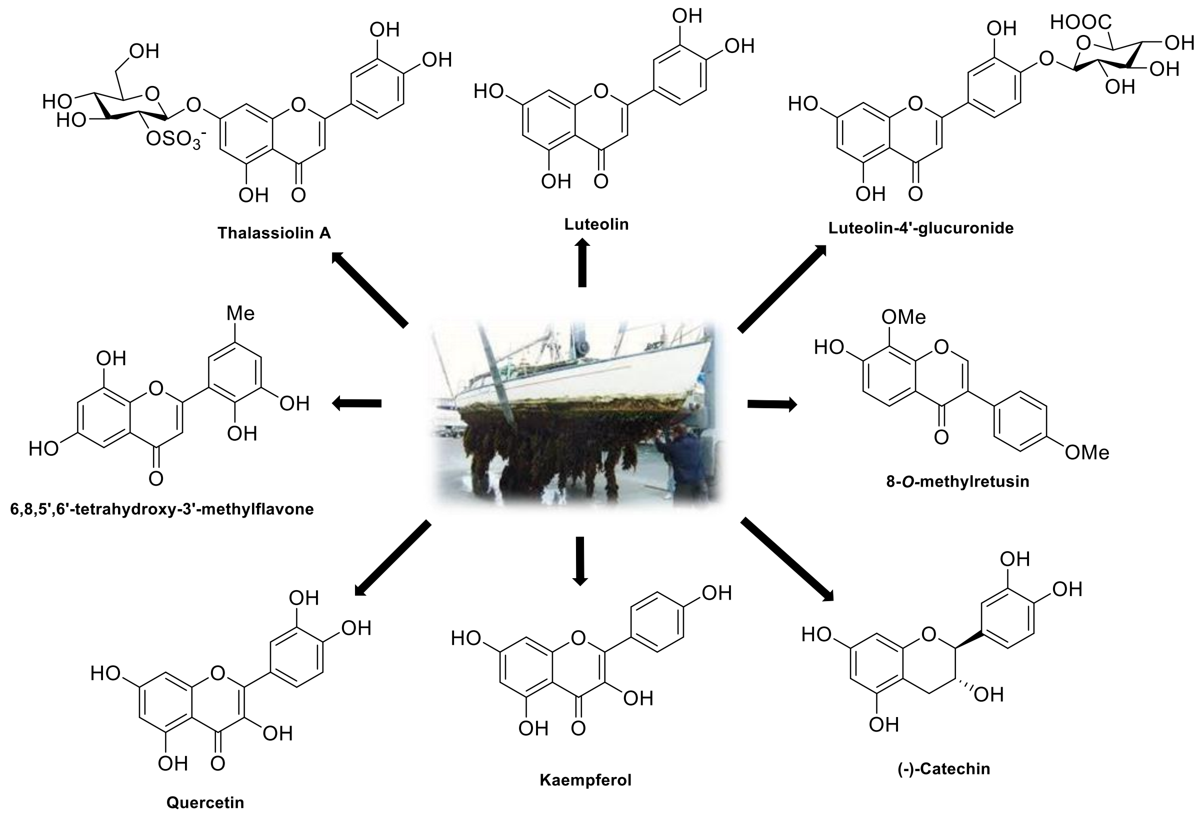


Figure 4 – Flavonoids with antifouling activity.

4. Synthesis of marine inspired flavones

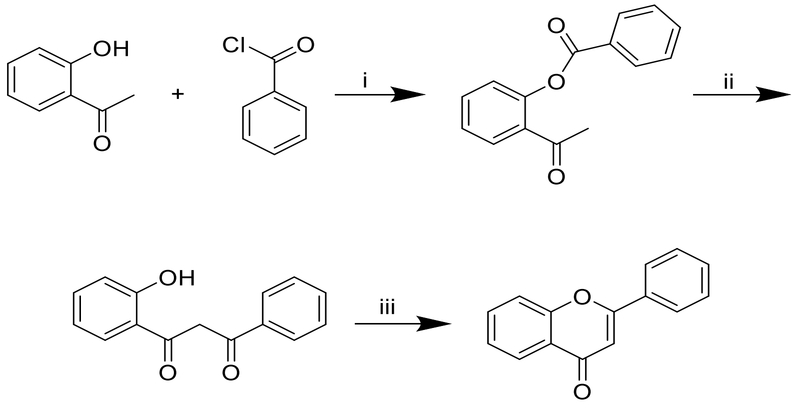
Flavonoids represent a well-known family of compounds and the synthesis of natural mimic flavonoids as well as new structure related compounds has been the object of a great number of studies. As the developed research work focus on the synthesis of marine inspired sulfated flavones, the main methodologies used for the total synthesis of this class of flavonoids, as well as the synthetic methods to obtain glycosylated and sulfated derivatives will be presented.

4.1. Synthesis of the flavone scaffold

The synthesis of flavonoid compounds, in particular flavones, has been well-reported in the literature. Several synthetic methods have been developed, namely Baker-Venkataraman-rearrangement, ionic liquid promoted synthesis, Vilsmeier-Haack reaction, Claisen-Schmidt condensation, Wittig reaction, solvent-free synthesis, and Suzuki-Miyaura reaction.

4.1.1. Baker–Venkataraman method

Most of the flavones are synthesized based on the Baker-Venkataraman method. It involves the conversion of 2’-hydroxyacetophenone into benzoyl ester, which undergoes an intramolecular Claisen condensation in the presence of a strong base (KOH/pyridine(Py)) to form β-diketone, which is cyclized to flavones by an acid-catalyzed cyclodehydration (**Scheme 1**). However, these reactions require the use of strong bases and long reaction time, being associated to low yields.[21]



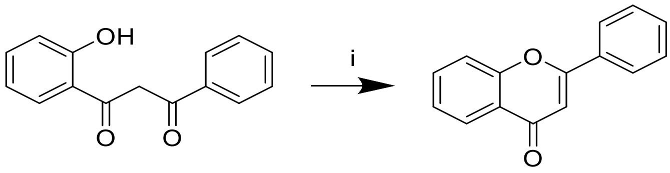
Scheme 1- Synthesis of flavones *via* β-diketone. Reaction conditions (i): Py; (ii): KOH, Py; (iii): AcOH glacial, H_2SO4 concentrate.

Alternatively, to the acid-catalyzed cyclodehydration promoted by AcOH glacial/H_2SO_4 various reaction conditions methods have been used, namely the use of Amberlyst, CoIII(sulfur)OH, $FeCl_3$, $Br_2/CHCl_3$, EtOH/HCl, clay, NaOAc/AcOH, and H_2SO_4 under microwave (MW) irradiation.[22] Additionally, the use of ionic liquids as a catalyst have been reported, as described in the next section.

4.1.2. Ionic liquid promoted synthesis

Various reactions have been reported recently using ionic liquids as a catalyst, due to their mild reaction conditions, short reaction times and better yield, solvating ability, and easy recyclability. They are salts of organic heterocyclic cations and inorganic anions and they exist in liquid state at ambient temperature, hence the reactions in presence of ionic liquids need no additional solvent.[22]

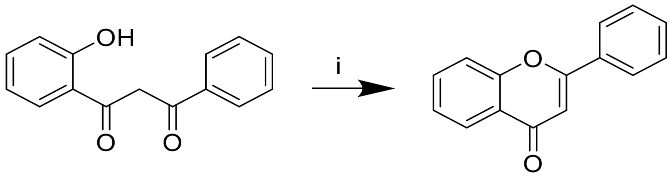
An example of the use of this ionic liquids to the synthesis of flavones in excellent yield with shorter reaction time was developed by Sarda *et al.,*[22] using the ionic liquid ethyl ammonium nitrate [$EtNH_3$]NO_3 as catalyst under MW irradiation to promote the cyclodehydration of β-diketone into flavone (**Scheme 2**).[22]



Scheme 2- Ionic liquid promoted synthesis. Reaction conditions (i): [$EtNH_3$]NO_3, MW, 22-50 sec.

4.1.3. Vilsmeier-Haack reaction

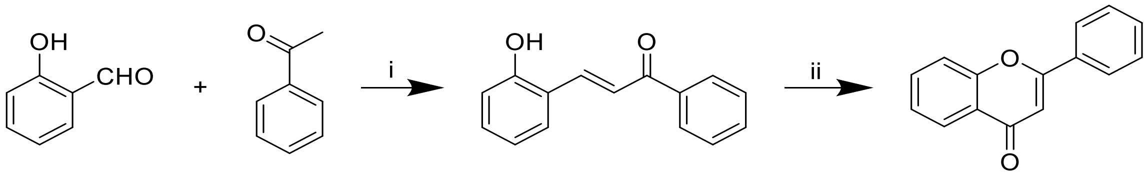
Vilsmeier-Haack reaction with *bis*-(trichloromethyl)carbonate/dimethylformamide (BTC/DMF) gives an efficient and rapid synthesis of flavones, by the cyclodehydration of the corresponding 1-(2-hydroxyphenyl)-3-aryl-1,3-propanediones (**Scheme 3**). This procedure has notable advantages such as high yields, mild reaction conditions, and short reaction times.[23]



Scheme 3 - Vilsmeier-Haack reaction. Reaction conditions (i): BTC/DMF.

4.1.4. Claisen-Schmidt condensation

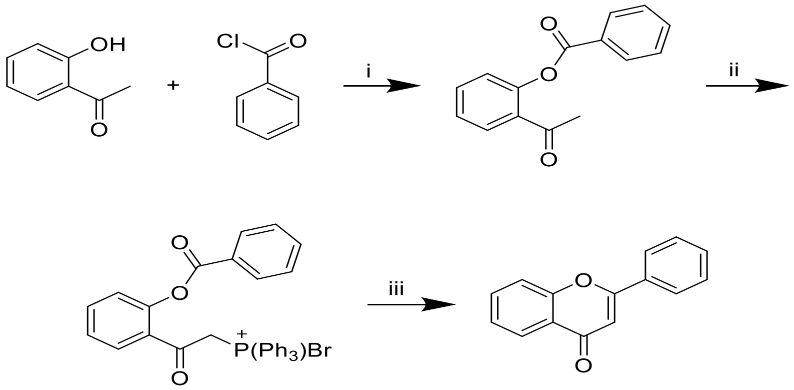
Flavones can also be synthesized by oxidative cyclization of 2'-hydroxylchalcones, derived from base-catalysed aldol condensation, or other catalysts, between appropriately substituted 2'-hydroxyacetophenones and benzaldehydes.[24] The aldol condensation represents an attractive reaction, since it predominantly generates the (*E*)-isomer, normally in high yield from commercially available building blocks.[25] Afterward, the oxidative cyclization of 2'-hydroxylchalcones afford the desired corresponding flavone by the refluxe in the presence of a diversity of reagents,[26] such as SeO_2 in dioxane, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ),[27] phenyliodine (III) diacetate (PIDA), diphenyl disulphide,[28] and catalytic amount of iodine in dimethylsulfoxide.[29] Several approaches for oxidative cyclization of 2'-hydroxylchalcones have been used to the classic methodologies, namely the use of solvent-free environmental friendly conditions (**Scheme 4**).[24]



Scheme 4- Claisen-Schmidt condensation reaction. Reaction conditions (i): 10% aqueous KOH, ethanol, reflux 2.5 h; (ii): I_2, 110-130 °C, 1.5 h.

4.1.5. Wittig reaction

The Wittig reaction utilizes 2′-hydroxyacetophenones, as the starting material, which are acylated with appropriate benzoyl chloride followed by reaction with bromine triphenylphosphine giving the corresponding phosphonium bromide, which then undergoes ring closure *via* intramolecular olefination of ester carbonyl group to afford the flavone (**Scheme 5**).[30-31]



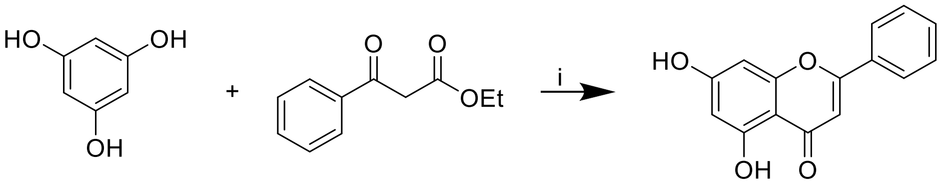
Scheme 5- Wittig reaction. Reaction conditions (i): Py; (ii): 1) Br_2, 2) PPh_3;

(iii): 1)Na_2CO_3, CH_2Cl_2, 2) NaOH, dioxane.

4.1.6. Solvent-free synthesis

Alternatively to classic methods, several eco-friendly methods have been developed to the synthesis of flavones. Mentzer *et al.* reported the direct solvent-free synthesis of flavones by the direct thermal cyclocondensation of phloroglucinol and *β*-ketoesters.[32] Seijas *et al.* reported the same reaction using MW irradiation, instead of conventional heating. Heating with microwaves instead of conventional heating was shown to be a higher yielding, cleaner, and faster approach. The reaction goes through a cycloaddition of an α-oxo ketene intermediate followed by an uncatalyzed thermal Fries rearrangement (**Scheme 6**).

The experimental setup is simple, just mixing the phenol and the *β*-ketoester and irradiating with microwaves without any solvent or solid. Reactions can be performed either under wattage control or temperature control (240 °C) with no significant differences in reaction times or yields.[33]

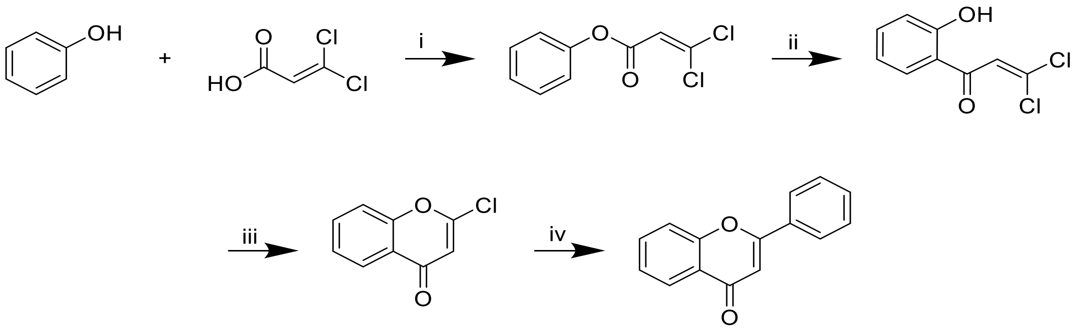


Scheme 6- Solvent-free synthesis. Reaction conditions (i): MW irradiation, 2-12 h.

Et = Ethyl group.

4.1.7. Suzuki-Miyaura reaction

Suzuki-Miyaura reaction involves insertion of palladium into a *sp*^{2}-hybridized C-X bond, being used for the synthesis of several flavonoids, including the flavone nucleus.[34] The main challenge with this procedure is the difficulty of accessing 2-halochromones, the precursors for the synthesis of flavones by Suzuki-Miyaura reaction. 2-Chlorochromone can be prepared from a sequence that involved esterification of phenol with 3,3-dichloroacrylic acid, followed by a Fries rearrangement and base-catalyzed cycloelimination. The reaction of 2-chlorochromone with boronic acids, under standard Suzuki conditions originate the flavones (**Scheme 7**).[34]



Scheme 7- Suzuki-Miyaura reaction. Reaction conditions (i): 1) DCC, DMAP, 2) CH_2Cl_2/DMF; (ii): 1) $AlCl_3$, 2) $Cl(CH_2)Cl$; (iii): 1) NaOH, 2) THF; (iv): 1) $Pd(PPh_3)_4$, 2) K_2CO_3, Dioxane.

4.2. Glycosylation

In nature, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be glucose, galactose, rhamnose, fucose, xylose or arabinose.[11, 30]

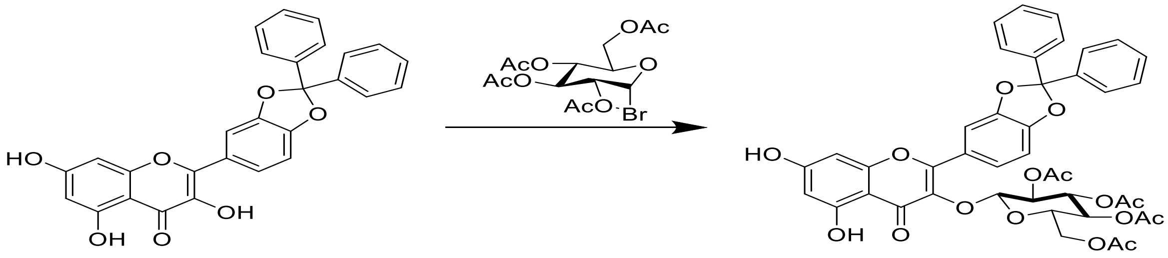
Flavonoid *O*-glycosides account for the majority of the extensive family of flavonoid glycosides and the majority of flavonoids have been synthesized by direct glycosylation of the flavonic scaffold with sugar donors, due to the susceptible properties of the *O*-glycosidic linkages.[35]

As most of the natural flavonoid glycosides are monoglycosylated polyphenols, the glycosylation of only one phenolic group is a challenge, obligating to protection and deprotection reactions. The most reported protecting groups are benzyl and acyl groups. Although flavonoid glycosides are stable under hydrogenolytic conditions, the introduction of benzyl groups onto flavonoids does not sufficiently enhance their solubility in conventional glycosylation solvents. Acyl groups, especially those bearing a relatively long alkyl chain, can increase the solubility of flavonoids. However, the basic conditions required for their removal can sometimes cause decomposition of the flavonoid derivatives. In addition, the electron-withdrawing effect of acyl groups decreases the reactivity of the phenolic hydroxyl groups of the flavonoid aglycone, making the glycosylation more difficult. Therefore, the choice of the protecting group has profound impact on the efficiency of the synthesis.[35]

There are a variety of glycosylation donors, including glycosyl bromides, chlorides, fluorides, imidates, and glycosyl esters which have been applied successfully in the synthesis of flavonoid glycosides. However, there is no universal method that could enable the highly efficient construction of all kinds of glycosidic linkages.[35]

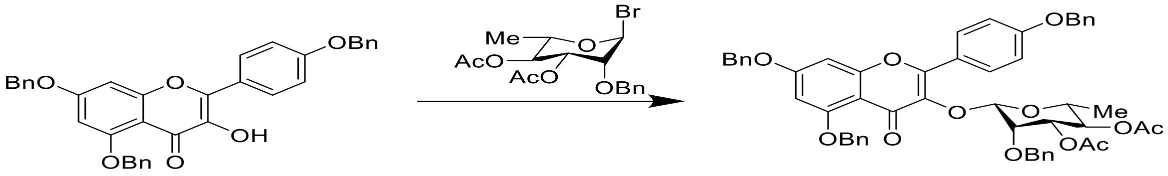
Glycosyl bromides are the most frequently used donor to be applied in the synthesis of flavonoid glycosides and three protocols are used for glycosylation with glycosyl bromide donors: the Zemplen–Farkas protocol, the Koenigs–Knorr protocol, and the phase-transfer-catalyzed (PTC) protocol.[35]

The Zemplen–Farkas protocol features the use of bases in a homogeneous medium, however has low yield due to the partial hydrolysis of the donor and the decomposition of the base-sensitive flavonoids. This protocol has been applied to the synthesis of quercetin 3-*O*-glucuronide by Bouktaib *et al.*[36] The glycosylation between 3’,4’-*O*-protected flavonoid and peracetyl glucosyl α-bromide was performed in the presence of potassium carbonate (K_2CO_3) in N,N-dimethylformamide (DMF) yielding 3-*O*-glucosylated quercetin derivative as the main product. Then, benzylation of the free phenolic hydroxyl groups and full deacetylation of the sugar moiety were performed (**Scheme 8**).[35-36]



Scheme 8- Zemplen–Farkas protocol. Reaction conditions : K_2CO_3, DMF.

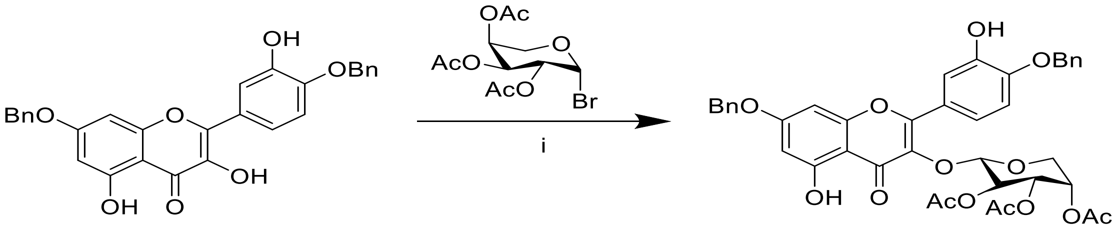
The Koenigs–Knorr protocol was originally developed for the glycosylation of alkyl hydroxyl groups with promotion by a silver salt, and this method has been successfully applied to the synthesis of some flavonoid glycosides, such as the kaempferol glycoside SL0101. The condensation of benzylated flavonoid with rhamnosyl bromide *via* the Koenigs–Knorr protocol (**Scheme 9**), afforded flavonol 3-*O*-glycoside, which was further debenzylated yielding kaempferol glycoside SL0101.[35, 37]



Scheme 9- Koenigs–Knorr protocol. Reaction conditions (i): Ag_2O, CH_2Cl_2;

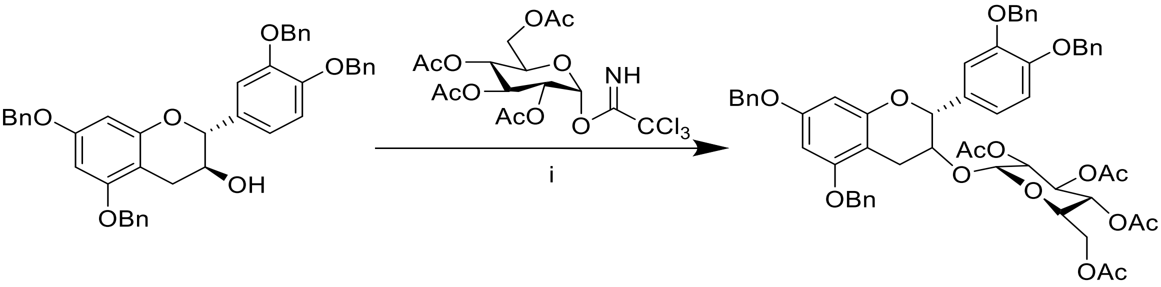
The poor solubility of the partially protected flavonoid acceptors in favourable glycosylation solvents (e.g., dichloromethane, diethyl ether, and toluene) demanded the use of highly polar and basic solvents such as quinolone and pyridine. However, using these solvents, the glycosylation yields are low and the workup processes is tedious. On the other hand, appropriately protected flavonoid acceptors, which have good solubility in conventional glycosylation solvents, could be glycosylated in satisfactory yields under Koenigs–Knorr conditions.[35] In addition, the need for more than one equivalent of the silver salt to promote the reaction bring about serious environmental problems when the reaction is conducted on a large scale.

The PTC method has overcome the disadvantages of Zemplen–Farkas and Koenigs–Knorr protocols. In the PTC method, flavonoid glycosides can be obtained by the reaction of flavonoids with glycosyl bromides in the two-phase solvent of chloroform/aqueous potassium carbonate, using tetrabutylammonium bromide (TBAB) as the phase-transfer catalyst (**Scheme 10**).[35, 38]



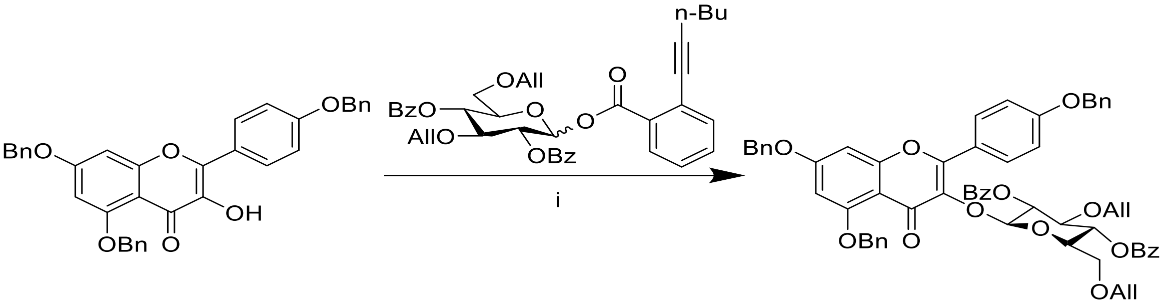
Scheme 10 - PTC protocol. Reaction conditions (i): TBAB, K_2CO_3 aqueous, $CHCl_3$, 40 °C. Ac = Acetyl group. Bn = Benzyl group.

Glycosyl trichloroacetimidates (TCA) have been applied in the synthesis of flavonoid glycosides, as the most prevailing glycosyl donors in synthetic carbohydrate chemistry. Thus, the glycosidic linkage of flavonoid 3-*O*-glycoside was produced by condensation of catechin with peracetylglucose *α*-TCA donor, with benzyl/silyl as protecting group (**Scheme 11**).[35, 39]



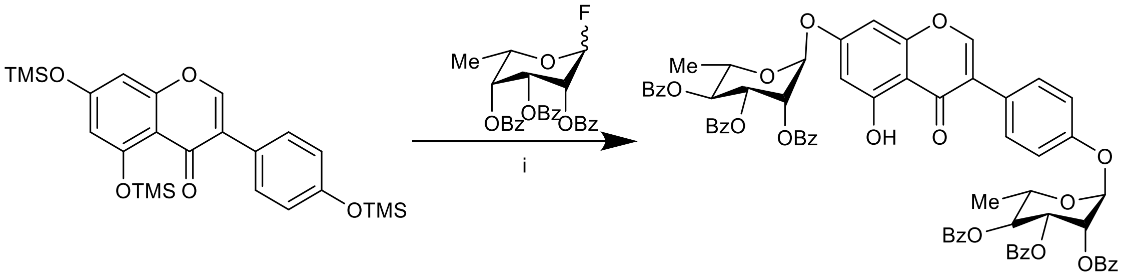
Scheme 11- Glycosylation with glycosyl trichloroacetimidates. Reaction conditions (i): TMSOTf, CH_2Cl_2, -40 °C. Ac = Acetyl group. Bn = Benzyl group.

In glycosylation with glycosyl esters, glycosyl *ortho*-alkynylbenzoates can be used as donors and under the catalysis of $Ph_3PAuNTf_2$, the glycosylation between the protected flavonoid and donor led to the desired glycoside (**Scheme 12**).[35, 40]



Scheme 12- Glycosylation with glycosyl esters. Reaction conditions (i): $Ph_3PAuNTf_2$, CH_2Cl_2. All = Alkynyl group. Bn = Benzyl group; Bz = Benzoyl group.

In glycosylation with glycosyl fluorides, the condensation of talopyranosyl fluoride, promoted by boron trifluoride, with the protected flavonoid provides 7,4’-*O*-diglycoside (**Scheme 13**).[35, 41]



Scheme 13- Glycosylation with glycosyl fluoride. Reaction conditions (i): $BF_3{\cdot}OEt_2$, CH_2Cl_2, 0 °C. TMS = Trimethylsilyl group; Bz = Benzoyl group.

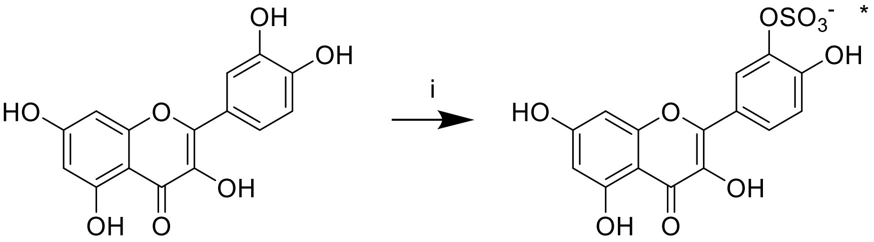
4.3. Sulfation

Flavonoids are the phenolic small molecules with more sulfated derivatives described, mostly within the flavonol and flavone subclasses. Although sulfation is a one-step reaction, synthesis of sulfated small molecules is quite challenging due the isolation of polysulfated compounds from the reaction mixtures, since the formation of a sulfate group drastically alters the physicochemical properties of a molecule.[42]

Some reagents, such as sulfamic acid (NH_2SO_2OH), sulfuric acid-derivatives or tetrabutylammonium hydrogen sulfate (TBAHS), have been utilized for the synthesis of sulfated flavonoids.[43]

Harborne’s group [44] used sulfamic acid in pyridine, in reflux, to sulfate several 3’-hydroxylflavonoids. This method allowed the introduction of a sulfate group through a pyridinium sulfamate intermediate, in the 3’-position, the least acidic position (**Scheme 14**).

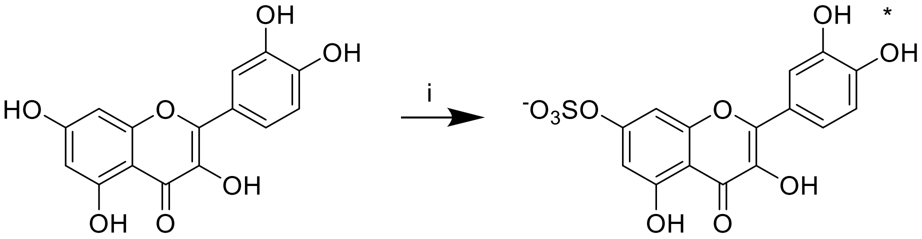
However, the most frequently found positions of the sulfate group in natural compounds are the 3- and/or 7-positions, so this method has little utility.[42]



Scheme 14- Sulfation with sulfamic acid. Reaction conditions (i): NH_2SO_2OH, Py, reflux. * - Major product.

Barron and Ibrahim attempted sulfation with sulfuric acid and dicyclohexylcarbodiimide (DCC) in DMF but this reaction failed as DCC was degraded before any sulfate derivative was formed.[42-43, 45]

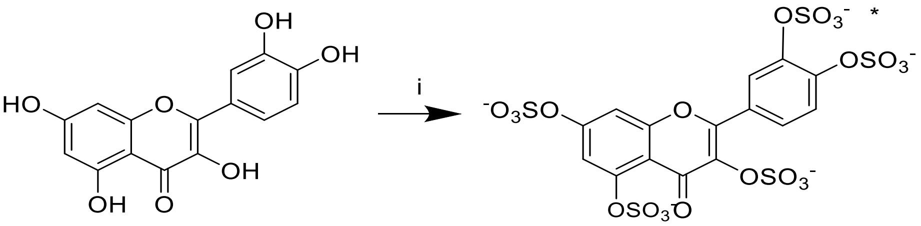
Alternatively, the DCC-mediated sulfation of several flavonoids with TBAHS, instead of sulfuric acid, has resulted in the formation of tetrabutylammonium salts of sulfated compounds at specific positions, being this method consequently widely used to obtain monosulfated compounds (**Scheme 15**).[45] Depending on the amount of the reagent used and the temperature of reaction, the established sequence of sulfation was 7> 4’ > 3> 3’, which could prove to be useful for the synthesis of the naturally occurring 7-sulfated flavones.[42-43]



Scheme 15- Sulfation with TBAHS/DCC. Reaction conditions (i): TBAHS, DCC, Py, 4 °C, 5 days. * - Major product.

In contrast with methods that use sulfuric acid-derivatives or TBAHS, sulfur trioxide adducts furnished products with high degree of substitution and low degradation. They result from the combination of SO_3, an acceptor of electrons or a Lewis acid, with a donor of electrons or Lewis bases.[42]

Adducts of SO_3 with organic bases, including Py, trimethylamine (TMA) (**Scheme 16**), and triethylamine (TEA), or amides such as DMF, have in general been found extensive use and have been replacing the sulfuric acid derivatives. The stability of the adduct directly varies with the force of the base used and inversely with its reactivity. Thus, SO_3·Py is the most reactive adduct and SO_3·TMA the least reactive one. However, even the most reactive adduct is a much softer reagent than free SO_3 or chlorosulfonic acid alone.[42]



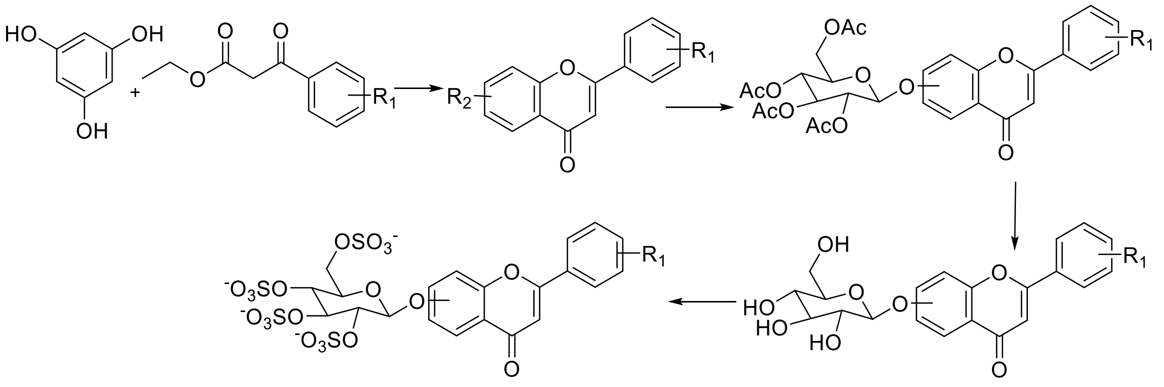
Scheme 16 - Sulfation with adducts of SO_3. Reaction conditions (i): SO_3·TEA, DMA, 65 °C, 3 h. * - Major product.

An additional problem of sulfated compounds, from a chemical perspective, is the lack of maneuverability following introduction of sulfate groups. Few functional group transformations can be successfully performed in the presence of sulfate group, which essentially forces the design of an adequate synthetic strategy to include sulfation as the final step.[42]

More selective strategies to warrant regioselectivity in sulfation reactions have been applied by enzymatic biosynthesis, which consists of incubation of the flavonoids substrates with sulfotransferases and the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The sulfation reaction corresponds to a nucleophilic substitution in which the transfer of sulfonate group is carried out through the nucleophilic attack on the sulfur atom of the PAPS sulfate group.[42]

5. Aims and research plan

The main purpose of this project was to obtain new flavonoids based on promising MNP leading to innovative antifouling agents. Inspired by the potential of marine natural flavonoids as non-toxic antifouling agents, namely thalassiolin A (**Figure 4, Pag. 10**), a new sulfated glycosylated flavone we aimed to synthesize structure related analogues. To fulfill this, four different flavones (**LuMe**, **TriCe**, **LuNi**, and **LuCl**) will be synthesized and afterward used as building blocks to obtain the correspondent sulfated glycosides as described in **Scheme 17**.



Scheme 17 – Research plan to obtain sulfated glycosides.

Other aims of this research work were:

- to apply classical and non-classical methodologies of synthesis;
- to elucidate the structure of the obtained derivatives through several spectroscopic techniques, namely IR and NMR (^{1}H, ^{13}C, HSQC, and HMBC);
- to evaluate the antifouling activity of the synthesized flavone derivatives, as well as the activity of a small library of other structure related flavonoids previously synthesized by LQOF/CIIMAR research group;
- to draw some considerations about structure-activity relationship (SAR);
- to predict the biodegradability and bioaccumulation in the aquatic environment of the most promising antifouling compounds using *in silico* methods.

CHAPTER II

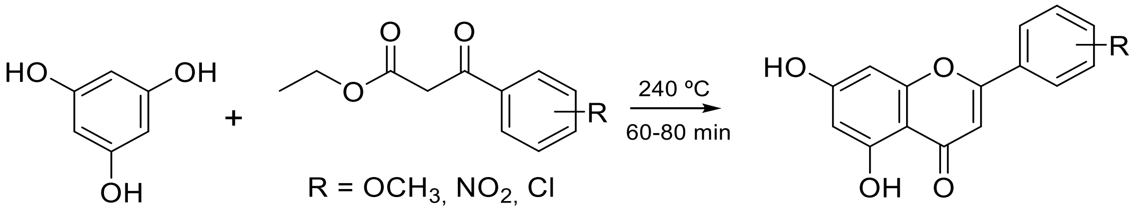
Results and Discussion

2.1. Synthesis

2.1.1. Flavone 7-*O*-acetyl glycosides

2.1.1.1. Flavones LuMe, TriCe, LuNi, and LuCl

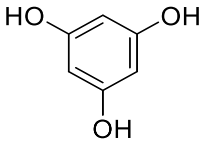
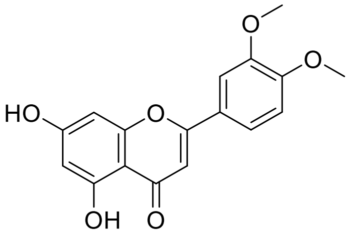
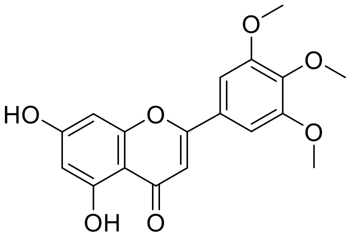
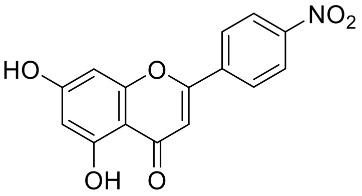
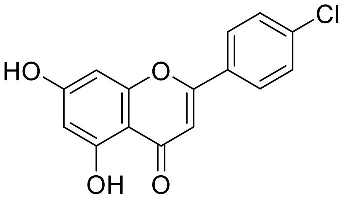
As described before, there are several synthetic methods for obtaining the flavone nucleus (**section 4.1.**). Among the various synthetic methodologies that are available, in this work, the building blocks luteolin 3',4'-dimethyl ether (**LuMe**), tricetin trimethyl ether (**TriCe**), 5,7-dihydroxy-4'-nitroflavone (**LuNi**), and 5,7-dihydroxy-4'-chloroflavone (**LuCl**) were obtained by solvent-free direct thermal cyclocondensation, the Mentzer synthesis.[32-33] This method is based on the reaction of phloroglucinol with 2 equivalents of *β*-ketoester, under 240 ºC in the muffle furnace (**Scheme 18**).



Scheme 18- General conditions for the Mentzer synthesis.

Table 1 summarizes the reaction conditions and the results obtained in the synthesis of flavone derivatives **LuMe**, **TriCe**, **LuNi**, and **LuCl**.

Table 1- General conditions for the synthesis of flavone derivatives.

Building block	Reaction conditions	Product	Yield
 Phloroglucinol	ethyl 3,4-dimethoxybenzoylacetate, 60 min	 **LuMe**	74%
	ethyl 3,4,5-trimethoxybenzoylacetate, 100 min	 **TriCe**	77%
	ethyl 4-nitrobenzoylacetate, 50 min	 **LuNi**	16%
	ethyl 4-chlorobenzoylacetate, 80 min	 **LuCl**	21%

The reaction yields for derivatives **LuMe** and **TriCe** were high, because only the desired product was obtained in the crude mixture, being not necessary to perform additional purification techniques. Nevertheless, derivatives **LuNi** and **LuCl** were obtained in lower yields. This could be explained by the fact that these reactions were not complete and led to the formation of several by-products. Therefore, the purification was necessary to be accomplished by flash column chromatography followed by crystallization.

These compounds were previously synthesized by Seijas *et al.*,[33] using MW irradiation instead of heating in a muffle furnace. Although the yields obtained for **LuMe** and **TriCe** were similar to those reported by Seijas,[33] the yields for the synthesis of **LuNi** and **LuCl** derivatives were lower, being this associated to the need of additional purification of compounds by column chromatography.

2.1.1.2. Flavone *O*-acetyl glycosides LuMeGluAc and TriCeGluAc

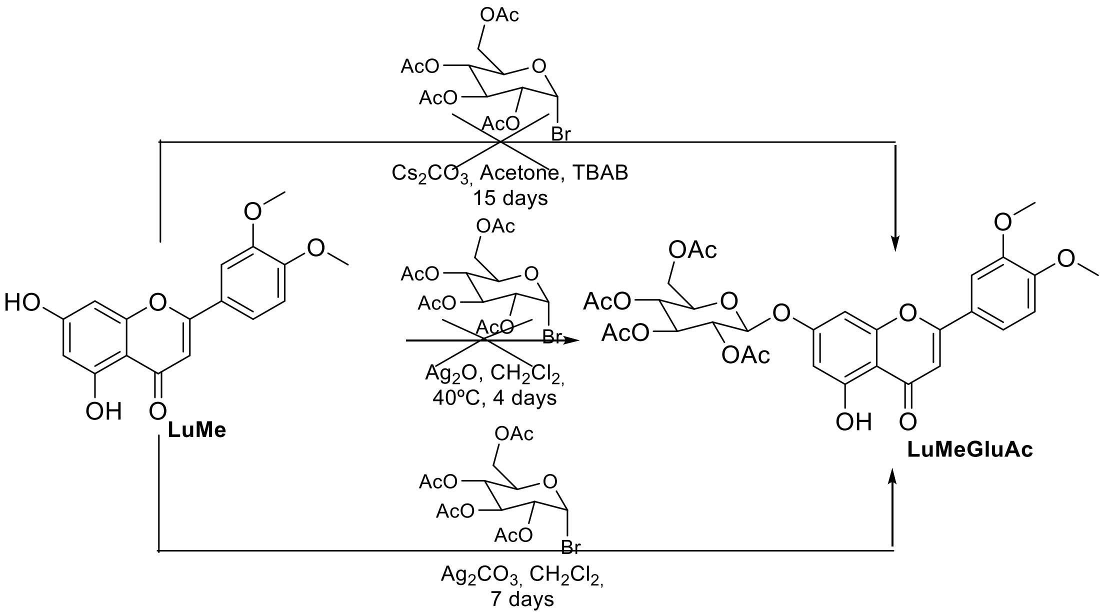
Glycosylated flavonoids are very common in marine world and have shown many beneficial effects on human health. In fact, many flavonoid glycosides have demonstrated a wide spectrum of biological activities, namely antifouling activity.[17-20]

As referred in **section 4.2.**, glycosyl bromides are the main common donor applied in flavonoid glycosides synthesis. After eight decades of development, various protocols are used for glycosylation with these donors: Zemplen–Farkas, Koenigs–Knorr, and Michael.[35] Considering the LQOF/CIIMAR experience,[46] in this work Michael method was firstly selected to be used for the synthesis of **LuMe** *O*-acetyl glycoside (**LuMeGluAc**). This derivative was prepared by the reaction of **LuMe** with acetobromo-*α*-D-glucose, in the presence of cesium carbonate (Cs_2CO_3), and TBAB in acetone (**Scheme 19**). However, this synthetic approach failed to produce the desired *O*-acetyl glycoside derivative, even after 15 days of reaction. More specifically, it has been found that this glycosylation reaction was not complete and led to the formation of a wide variety of by-products which made impossible to obtain the desired *O*-acetyl glycoside derivative.

In order to afford this derivative, successive attempts of purification were carried out. Firstly, the reaction mixture was purified by flash column chromatography using n-hexane/ethyl acetate as eluent. No pure fractions were obtained, and therefore, the fractions that contained the same chromatographic profile were joined and, subsequently, purified by preparative thin layer chromatography (TLC). Although all these purification steps have been performed, the desired **LuMeGluAc** was not isolated.

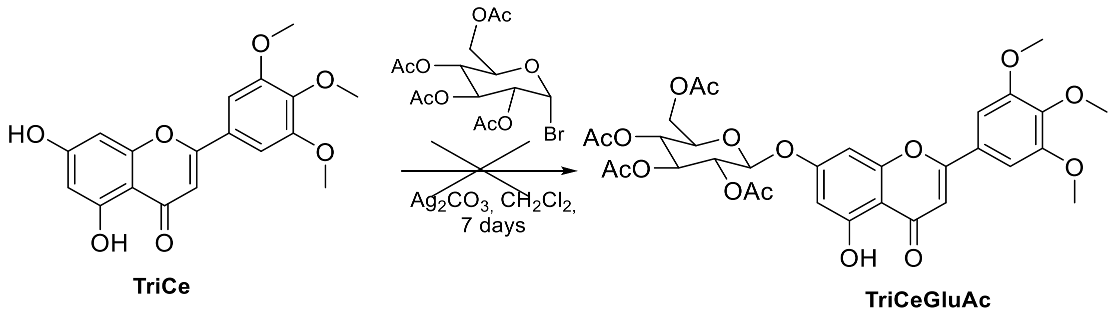
To overcome the difficulties experienced with the use of the Michael protocol, it was attempted the glycosylation using the silver salts according to the Koenigs–Knorr protocol. Therefore, a reaction between **LuMe** and acetobromo-*α*-D-glucose in the presence of silver oxide (Ag_2O) in dichloromethane during 4 days, at 40 °C was performed (**Scheme 19**).[47] However, this synthetic approach also failed to produce the desired *O*-acetyl glycoside derivative, with the same problems as Michael protocol.

Consequently, a similar reaction was attempted with another silver salt, silver carbonate (Ag_2CO_3), as described by Needs and Williamson.[48] Using this approach, the treatment of **LuMe** in dicloromethane with acetobromo-*α*-D-glucose and silver carbonate, for 7 days gave the desired *O*-acetyl glycoside derivative **LuMeGluAc** with low yield (7%) (**Scheme 19**). This low yield could be justified by the fact of this reaction has been incomplete and thus, the work-up was difficult and time-consuming. Particularly, the purification of **LuMeGluAc** by flash column chromatography, using n-hexane/ethyl acetate as eluent, was difficult because some by-products formed in this reaction had a similar polarity to the desired compound (**Scheme 19**).



Scheme 19- Different routes to the synthesis of **LuMeGluAc**.

The Koenigs–Knorr protocol with silver carbonate was also tried for glycosylation of **TriCe**. However, this synthetic approach failed to produce the **TriCeGluAc** (**Scheme 20**). More specifically, it has been found that this glycosylation reaction was not complete and led to the formation of a wide variety of by-products which made impossible to obtain the desired *O*-acetyl glycoside derivative. In order to afford this derivative, successive attempts of purification were carried out by crystallization and flash column chromatography, but no pure fractions were obtained.



Scheme 20- Synthesis of **TriCeGluAc**.

Despite these results not being expected, they are supported by the literature. A major disadvantage of glycosylation reactions is the formation of by-products that could cause difficulties in the purification of the final product. In fact, several side-reactions have been identified as the main routes which may be involved in the production of undesired by-products in glycosylation reactions. These main pathways are hydrolysis of the glycosyl donor and 1,2-elimination to give a glycal.[49-50] Also, many flavonoid derivatives are poorly soluble in glycosylation solvents and they are disposed to degradation under acidic and basic conditions.[35]

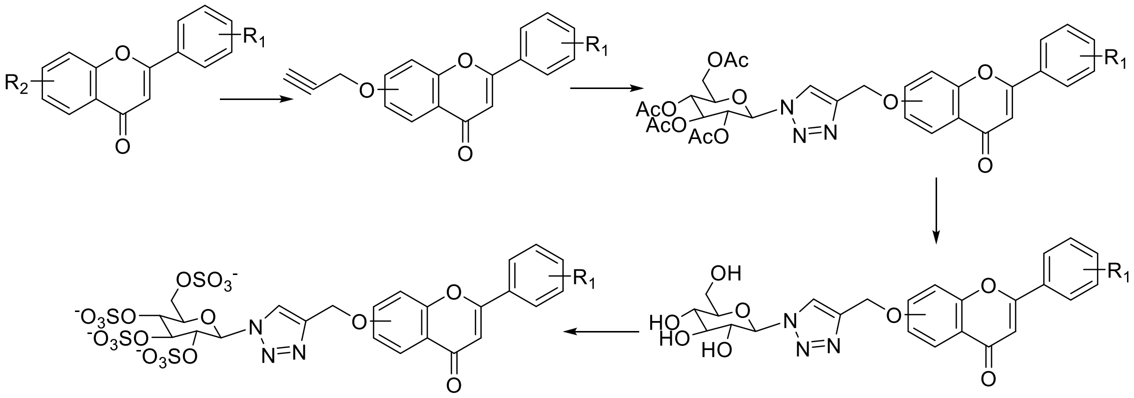
The low yield and the difficulties during the synthesis and purification processes of glycosylation reactions disallowed obtaining sulfated glycosylated flavone derivatives as firstly planned. To overcome these limitations another approach to introduce the glycosidic moiety by click chemistry was tried.

2.1.2. Glycosylated flavones by Click chemistry

Pioneered by Huisgen in the 1960s, the 1,3-dipolar cycloaddition reaction, also known as Huisgen cycloaddition, between an azide and a terminal alkyne affording the 1,2,3-triazole moiety, was brought back into focus by Sharpless and others when they developed the term of “click chemistry”.[51-52] This term was created to designate reactions defined by a set of stringent criteria: “The reaction must be modular, wide in scope, give very high yields, generate only inoffensive by-products that can be removed by non-cromatographic methods, and be stereospecific, but not necessarily enantioselective. The required process characteristics include simple reaction conditions, readily available starting materials and reagents, the use of no solvents or a solvent that is benign (such as water) or easily removed, and simple product isolation.”.[53]

Over the last decade, there has been a great interest in the synthesis of 1,2,3-triazole units due to the fact to these moieties are more than passive linkers. They have some favourable physicochemical properties and showed importance to biological activity.[54-55] In fact, this approach, is being used to generate a vast array of compounds with biological potential,[53, 56-58] namely with antifouling activity.[59-60] Moreover, many antimicrobial agents are based on nytrogen heterocycles, including triazole-based biocides, which suggest their potential to act as antifouling agents against the settlement of microfouling species.[59]

Considering this, it was planned to synthesize structure related flavones possessing a 1,2,3-triazole ring as a linker between the flavone nucleus and the sulfated glycosyl moiety using **LuMe**, **TriCe**, **LuNi**, and **LuCl** as building blocks (**Scheme 21**).

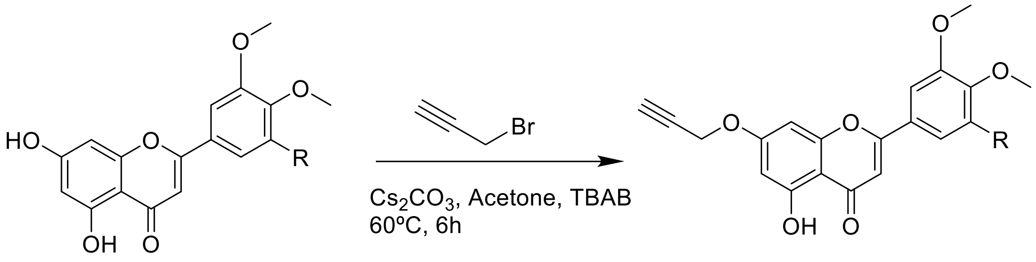


Scheme 21 -Research plan to obtain sulfated glycosides with triazole linker.

2.1.2.1. Propargyl flavone derivatives LuMeProp and TriCeProp

In view of their appropriateness for further transformations, propargylic derivatives of aromatic compounds are one of the most suitable motifs found in synthetic intermediates for the synthesis of pharmaceuticals and natural products.[61]

The first step in the synthetic process to obtain glycosylated flavones, was the propargylation of the previously prepared **LuMe** and **TriCe**. The synthetic approach used for the synthesis of **LuMeProp** and **TriCeProp** was based on the reaction of **LuMe/TriCe** with one equivalent of propargyl bromide, in the presence of anhydrous Cs_2CO_3 and TBAB, in reflux in acetone (**Scheme 22**).



R=H **LuMe**
R=OCH_3 **TriCe**

R=H **LuMeProp**
R=OCH_3 **TriCeProp**

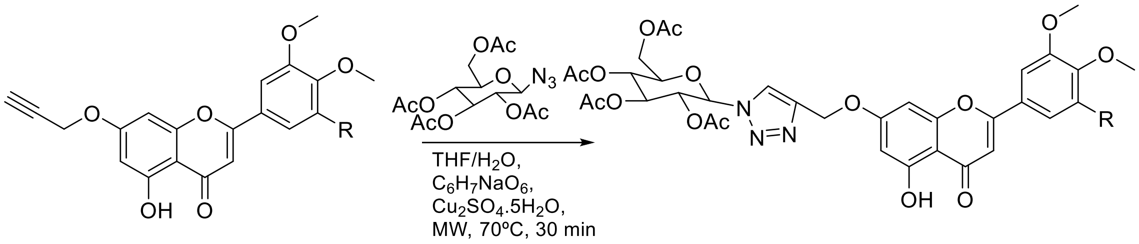
Scheme 22- Synthesis of **LuMeProp** and **TriCeProp**.

Since the hydroxyl group in position 5 is involved in an intramolecular hydrogen bond with carbonyl group, only the 7-*O*-monosubstituted derivatives were obtained with a 66% yield to **LuMeProp** and 55% yield to **TriCeProp**, after purification by flash column chromatography followed by crystallization in acetone. These yields are explained by the fact of the formation in considerable amounts of more than one product and the difficulties related with the purification by column chromatography.

2.1.2.2. Flavone *O*-acetyl glycosides LuMeTGluAc and TriCeTGluAc

LuMeTGluAc and **TriCeTGluAc** were synthesized by MW assisted Copper(I)-catalyzed azide alkyne cycloaddition (CuAAC). The reaction between the previously formed **LuMeProp/TriCeProp** and 2,3,4-tetra-*O*-acetyl-*β*-glucopyranosyl azide (previously synthesized in LQOF/CIIMAR research group) was carried out in the presence of copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) and sodium ascorbate ($C_6H_7NaO_6$), in a non-homogeneous mixture of water and tetrahydrofuran (THF) (1:2) for 30 minutes at 70 ºC under MW irradiation at 500 W (**Scheme 23**).

MW allows efficient internal heat transfer and hence reduces the reaction time as well as increasing the reaction rate and the yield. Elevated temperatures can be used over short periods (min) thus avoiding decomposition or polymerization.[54]



R=H **LuMeProp**
R=OCH_3 **TriCeProp**

R=H **LuMeTGluAc**
R=OCH_3 **TriCeTGluAc**

Scheme 23- Synthesis of **LuMeTGluAc** and **TriCeTGluAc**.

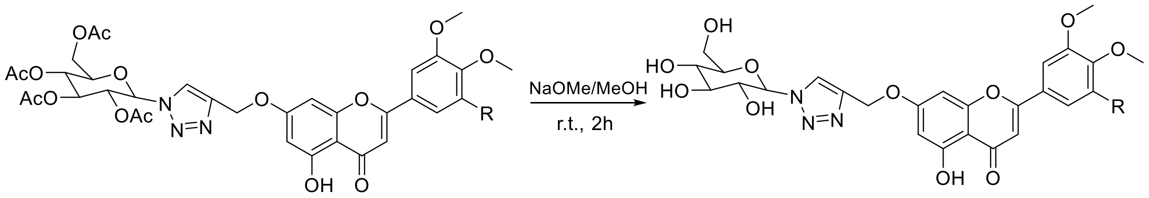
After, the resulting products were purified by crystallization with acetone. **LuMeTGluAc** was obtained with a 82% yield and **TriCeTGluAc** was obtained with a 79% yield.

2.1.2.3. Flavone *O*-glycosides LuMeTGluOH and TriCeTGluOH

Deprotection of the glycosidic moiety of flavone *O*-acetyl glycosides was achieved under Zemplén conditions, one of the most common deprotection strategies. This reaction, firstly reported by Zemplén and Kuntz in 1924, is an efficient removal of *O*-acetyl protecting groups of glycosidic moieties by treatment with a catalytic amount of sodium methoxide (NaOMe) in methanol at room temperature.[62]

Today, Zemplén deacetylation is a standard tool in laboratories but its inherent disadvantage is the retention in solution of sodium ions. These sodium ions can be removed using H^+-exchanged resin, in ion-exchanged columns, and the resin can be regenerated with acid after ion exchange.[63]

Flavone *O*-glycosides **LuMeTGluOH** and **TriCeTGluOH** were obtained after desprotection of **LuMeTGluAc** and **TriCeTGluAc**, respectively, with sodium methoxide, during 2 hours at room temperature (**Scheme 24**).



R=H **LuMeTGluAc**
R=OCH_3 **TriCeTGluAc**

R=H **LuMeTGluOH**
R=OCH_3 **TriCeTGluOH**

Scheme 24- Synthesis of **LuMeTGluOH** and **TriCeTGluOH**.

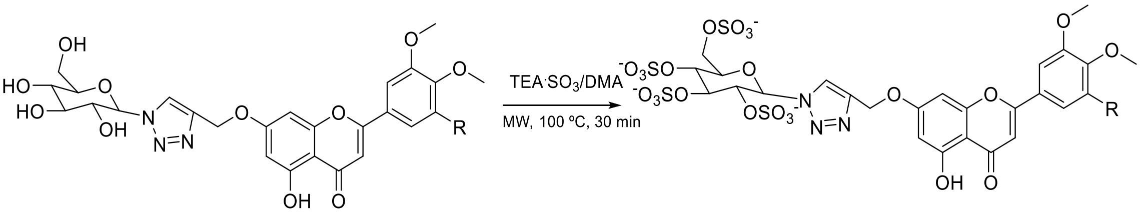
The flavone *O*-glycosides **LuMeTGluOH** and **TriCeTGluOH** were obtained with good yields, 85% and 77%, respectively. After reaction, the solutions were treated with H^+-exchanged resin, in order to remove the sodium ions.

2.1.2.4. Flavone *O*-sulfate glycosides LuMeTGluS and TriCeTGluS

Sulfated natural products have emerged as antifouling agents with low or non-toxic effects to the environment.[64] So, in order to proceed with the main objective of this research, the sulfation of flavone *O*-glycosides was performed.

As referred in **section 4.3.**, sulfation with sulfur trioxide adducts is extensively used, allowing to obtain sulfated compounds with a high degree of sulfation and low degradation.[42] However, the sulfated derivatives are difficult to isolate mainly due to the fact that they are water soluble, being necessary to remove water-soluble impurities by dialysis using a Spectra/Por 6 regenerated cellulose membrane (MWCO 1000).[65]

LuMeTGluS and **TriCeTGluS** were obtained by MW assisted sulfation of flavone *O*-glycosides **LuMeTGluOH** and **TriCeTGluOH** with triethylamine-sulfur trioxide adduct (TEA·SO_3) in DMA solution for 30 minutes at 100ºC under MW irradiation at 200 W, according to the LQOF/CIIMAR experience [66] (**Scheme 25**).



R=H **LuMeTGluOH**
R=OCH_3 **TriCeTGluOH**

R=H **LuMeTGluS**
R=OCH_3 **TriCeTGluS**

Scheme 25- Synthesis of **LuMeTGluS** and **TriCeTGluS**.

At the end, the reactions were treated with TEA to form TEA salts, which were then insolubilized, in the form of oil, with acetone, allowing the separation from the excess of adduct. The TEA salts were afterward converted into sodium salts by addition of sodium acetate (CH_3COONa) and insolubilized with ethanol. The obtained solid was further purified by dialysis using a Spectra/Por 6 regenerated cellulose membrane (MWCO 1000) during 1h to furnish the pure compounds **LuMeTGluS** and **TriCeTGluS** with 80% and 77% yield, respectively.

2.2. Structure elucidation

The structure elucidation of all flavones was established on the basis of IR, and NMR techniques. ^{13}C NMR assignments were determined by 2D heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments. The numbering concerning the NMR assignments is presented in **Figure 5**.

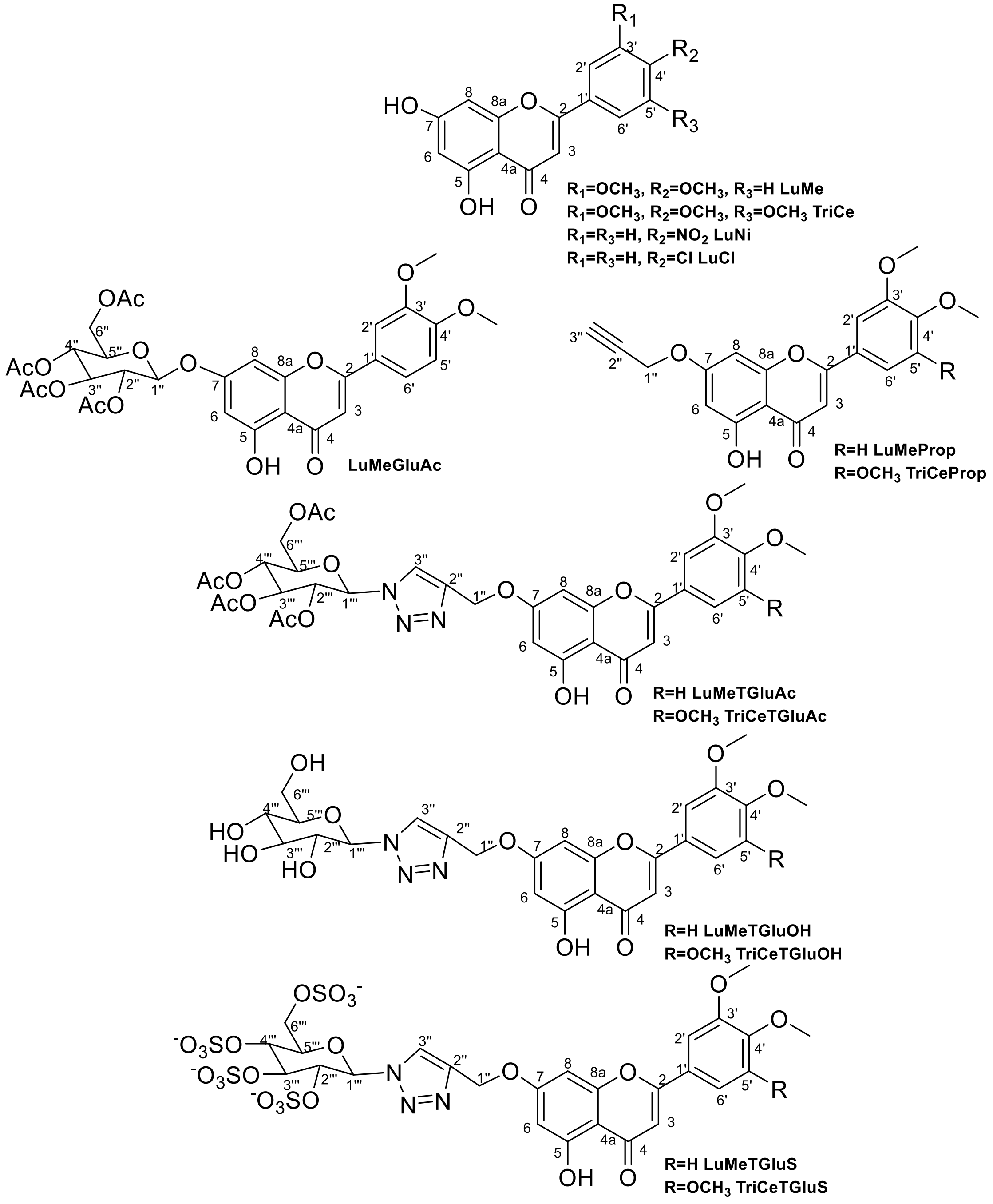


Figure 5 - The numbering of synthesized compounds concerning the NMR assignments.

2.2.1. Flavone derivatives LuMe, TriCe, LuNi, and LuCl

The IR data of flavone derivatives **LuMe**, **TriCe**, **LuNi**, and **LuCl** are presented in **Table 2**. Accordingly, the IR spectra showed the presence of absorption bands corresponding to hydroxyl (3600-3300 cm^{-1}), C=O (1665-1653 cm^{-1}), aromatic C=C (1598-1429 cm^{-1}), and C-O (1274-1256 cm^{-1}) groups. Additionally, for **LuMe** and **TriCe** bands at 2950 cm^{-1} and 2839 cm^{-1} suggested the presence of alkyl groups. For **LuNi** a band at 1372 cm^{-1} was visualized in accordance to the presence of a nitro group, and for **LuCl** a band at 785 cm^{-1} was observed suggesting the presence of a chlorine atom.

Table 2 – IR data of **LuMe, TriCe, LuNi,** and **LuCl**.

Groups	**υ (cm^{-1})**			
	LuMe	**TriCe**	**LuNi**	**LuCl**
OH	3600-3300	3600-3300	3600-3300	3600-3300
Aliphatic C-H	2950	2950 2839	-	-
C=O	1655	1655	1653	1655
Aromatic C=C	1581 1517 1504 1429	1592 1558 1505 1441	1598 1518 1456 1431	1586 1575 1510 1493
N-O	-	-	1372	-
C-O	1256	1257	1274	1269
C-Cl	-	-	-	785

The ^{1}H and ^{13}C NMR data of flavone derivatives **LuMe**, **TriCe**, **LuNi**, and **LuCl** are reported in **Tables 3** and **4**, respectively.

The ^{1}H NMR spectra of all flavone derivatives showed characteristic signals of a 5,7-dihydroxyphenyl A ring (OH-5: δ_H 12.92-12.53 s; H-6: δ_H 6.29-6.22 d; OH-7: δ_H 10.97-10.54 s; H-8: δ_H 6.59-6.49 d), and H-3 (δ_H 7.07-6.86 s).

For derivatives **LuNi** and **LuCl**, the ^{1}H NMR spectra also showed two *orto* coupled doublets at δ_H 8.38-8.16 and δ_H 8.09-7.63, respectively, characteristic of a 4'-substituted B ring. Alternatively, for derivatives **LuMe** and **TriCe** characteristic signals of a 3',4'-dimethoxy ($\delta_{H:}$ 7.68 dd, 7.56 d, 7.12 d, 3.88 s, 3.85 s; $\delta_{C:}$ 152.2, 149.0, 122.9, 120.1, 111.7, 109.4, 55.9, 55.8) or 3',4',5'-trimethoxy (δ_H 7.33 s, 3.90 s, 3.75 s; δ_C 153.3, 140.8, 126.0, 104.1, 60.3, 56.3) phenyl B ring are observed in ^{1}H and 13C NMR spectra, respectively.

The structure of all derivatives was confirmed by comparison with the ^{1}H and ^{13}C NMR data previously reported.[33]

Table 3- ^{1}H NMR data of flavone derivatives **LuMe**, **TriCe**, **LuNi**, and **LuCl**.

	LuMe	**TriCe**	**LuNi**	**LuCl**
H-3	6.96 (s)	7.07 (s)	6.86 (s)	7.00 (s)
H-5	12.92 (s, OH)	12.86 (s, OH)	12.53 (s, OH)	12.77 (s, OH)
H-6	6.23 (d, J=2.1)	6.23 (d, J=2.1)	6.29 (d, J=2.0)	6.22 (d, J=2.1)
H-7	10.97 (s, OH)	10.94 (s, OH)	10.54 (s, OH)	n.o.
H-8	6.55 (d, J=2.1)	6.59 (d, J=2.1)	6.49 (d, J=2.0)	6.52 (d, J=2.1)
H-2'	7.56 (d, J=2.1)	7.33 (s)	8.16 (d, J=8.9)	8.09 (d, J=9.2)
H-3'	3.85 (s, OCH_3)	3.90 (s, OCH_3)	8.38 (d, J=8.9)	7.63 (d, J=9.2)
H-4'	3.88 (s, OCH_3)	3.75 (s, OCH_3)	-	-
H-5'	7.12 (d, J=8.7)	3.90 (s, OCH_3)	8.38 (d, J=8.9)	7.63 (d, J=9.2)
H-6'	7.68 (dd, J=8.7; J=2.1)	7.33 (s)	8.16 (d, J=8.9)	8.09 (d, J=9.2)

n.o. not observed; Values in parts per million (δ_H). Measured in DMSO-d_6 at 300.13 MHz. J values (Hz) are presented in parentheses.

Table 4- ^{13}C NMR data of flavone derivatives **LuMe**, **TriCe**, **LuNi**, and **LuCl**.

	LuMe	**TriCe**	**LuNi**	**LuCl**
C-2	163.4	163.1	161.5	162.0
C-3	103.9	105.1	107.4	105.5
C-4	181.9	182.0	181.3	181.8
C-4a	103.8	103.9	104.2	104.0
C-5	161.4	161.4	160.3	161.5
C-6	99.0	99.0	99.3	99.1
C-7	164.3	164.4	164.6	164.5
C-8	94.2	94.4	94.0	94.2
C-8a	157.4	157.5	157.3	157.4
C-1’	122.9	126.0	136.7	129.6
C-2’	109.4	104.1	123.7	129.2
C-3’	149.0	153.3	127.1	128.3
C-4’	152.2	140.8	148.8	136.8
C-5’	111.7	153.3	127.1	128.3
C-6’	120.1	104.1	123.7	129.2
OCH_3	55.9, 55.8	60.3, 56.3	-	-

Values in parts per million (δ_C). Measured in DMSO-d_6 at 75.47 MHz.

2.2.2. Flavone *O*-acetyl glycoside LuMeGluAc

The IR of *O*-acetyl glycoside **LuMeGluAc** was in accordance with the performed molecular modification (**Table 5**). The presence of one free hydroxyl group was revealed by the presence of a large band of stretching vibration between 3600-3300 cm^{-1}, suggesting that glycosylation have occurred only on one hydroxyl group. Additionally, the presence of acetyl groups was suggested by the observation of a strong band at 1746 cm^{-1} typical of C=O ester stretch.

Table 5 - IR data of **LuMeGluAc**.

Groups	**υ (cm^{-1})**
	LuMeGluAc
OH	3600-3300
Aliphatic C-H	2963
C=O	
Ester	1746
Ketone	1647
Aromatic C=C	1603 1518 1505 1456
C-O	1261

The ^{1}H and ^{13}C NMR data of *O*-acetyl-*β*-glucopyranosyl derivative **LuMeGluAc** is reported in **Table 6**.

Characteristic signals of **LuMe** scaffold were observed in the ^{1}H and ^{13}C NMR spectra of *O*-acetyl glycoside **LuMeGluAc**. Nevertheless, instead of a singlet at δ_H 10.97 corresponding to OH-7, characteristic signals of an *O*-acetyl-*β*-glucopyranosyl group were observed in the ^{1}H and ^{13}C NMR spectra of derivative **LuMeGluAc**, confirming the substitution of this position. The position of this group was evidence by the correlations found in the HMBC spectra illustrated in **Figure 6**.

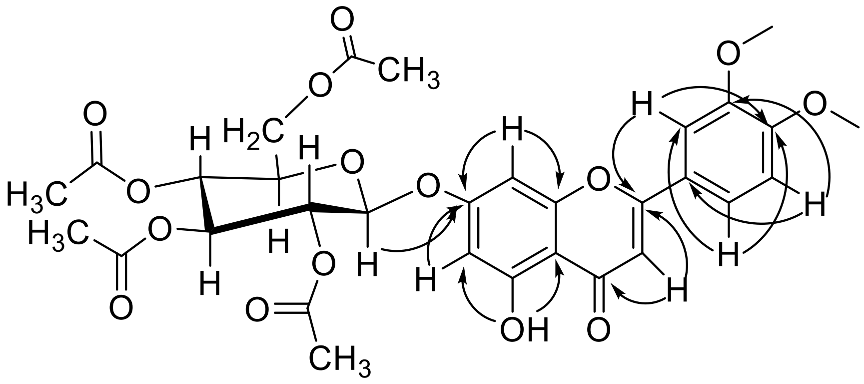


Figure 6– Main connectivities found in the HMBC of *O*-acetyl-*β*-glucopyranosyl derivative **LuMeGluAc**.

Table 6- ^{1}H and ^{13}C NMR data of derivative **LuMeGluAc**.

	LuMeGluAc	
	^{1}H	**^{13}C**
2	-	164.2
3	6.60 (s)	104.8
4	-	182.4
4a	-	104.3
5	12.81 (s, OH)	163.2
6	6.45 (d, J=2.1)	98.8
7	-	164.6
8	6.58 (d, J=2.1)	93.6
8a	-	163.0
1’	-	123.7
2’	7.34 (d, J=2.1)	108.8
3’	-	157.6
4’	-	162.2
5’	6.99 (d, J=8.7)	111.2
6’	7.53 (dd, J=8.7; J=2.1)	120.2

Table 6 (Contd.)- ^{1}H and ^{13}C NMR data of derivative **LuMeGluAc**.

	LuMeGluAc	
	^{1}H	**^{13}C**
1”	4.98 (d, J=9.0)	97.7
2”-6”	4.77-3.97 (m)	77.2, 76.9, 76.5, 74.2, 73.2
OCH_3	3.99 (s), 3.98 (s)	56.2, 56.1
$COCH_3$	2.19 (s), 2.08(s), 2.07 (s), 2.00 (s)	170.6, 170.1, 169.5, 169.2 ($\underline{C}$=O) 20.7, 20.6, 20.1, 19.8 ($O\underline{C}H_3$)

Values in parts per million (δ$_H$ and δ$_C$). Measured in $CDCl_3$ at 300.13 and 75.47 MHz. J values (Hz) are presented in parentheses.

2.2.3. Propargyl flavone derivatives LuMeProp and TriCeProp

The IR data of propargyl flavone derivatives **LuMeProp** and **TriCeProp** showed the characteristics signals of precursors **LuMe** and **TriCe** (**Table** 7). Additionally, bands at 2360 cm^{-1} (C≡C-H) suggested the presence of an alkyne moiety.

Table 7 – IR data of **LuMeProp** and **TriCeProp**.

Groups	**υ (cm^{-1})**	
	LuMeProp	**TriCeProp**
OH	3600-3300	3600-3300
Aliphatic C-H	2963 2917 2849	2919
C≡C-H	2360	2360

Table 7 (Contd.)– IR data of **LuMeProp** and **TriCeProp**.

Groups	**υ (cm^{-1})**	
	LuMeProp	**TriCeProp**
C=O	1647	1647
Aromatic C=C	1518 1504 1468 1453 1429	1596 1497 1456 1423
C-O	1261	1258

The ^{1}H and ^{13}C NMR data of propargyl derivatives **LuMeProp** and **TriCeProp** are reported in **Table 8**.

As expected, the ^{1}H and ^{13}C NMR spectra of both propargyl derivatives are very similar to those of the precursors. However, no characteristic signals of OH-7 are observed in the spectra ^{1}H NMR of **LuMeProp** and **TriCeProp**, suggesting that the substitution occurred in this position. Alternatively, signals of two oxymethylenic protons at δ 4.94-4.76 d (J=2.0 Hz, H-1") and one methynic proton at 3.68-3.61 brs (H-3"), correlated in the HSQC spectra with the ^{13}C NMR signals at δ_C 56.9, 56.7 (C-1") and δ_C 79.1, 78.4 (C-3"), respectively, were observed, suggesting the propargylation of the hydroxyl group at C-7 (**Figure** 7).

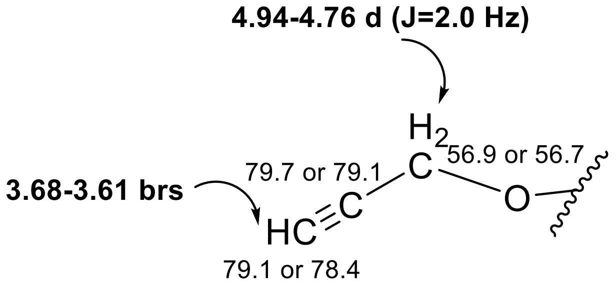
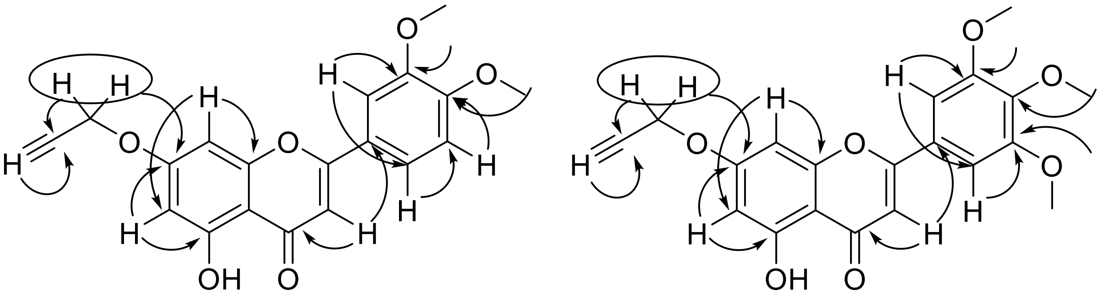


Figure 7- ^{1}H and ^{13}C NMR data for the propargyl group **of LuMeProp** and **TriCeProp**.

The position of the propargyl side chain was confirmed by the correlations found in the HMBC as indicated in **Figure 8**.



LuMeProp **TriCeProp**

Figure 8– Main connectivities found in the HMBC of **LuMeProp** and **TriCeProp**.

Table 8- ^{1}H and ^{13}C NMR data of propargyl flavone derivatives **LuMeProp** and **TriCeProp**.

	LuMeProp		**TriCeProp**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
2	-	163.7	-	163.1
3	6.84 (s)	105.8	7.15 (s)	105.3
4	-	182.8	-	182.2
4a	-	104.8	-	105.3
5	12.74 (s, OH)	161.8	12.85 (s, OH)	161.2
6	6.26 (d, J=2.0)	99.3	6.44 (d, J=2.1)	98.7
7	-	164.5	-	163.4
8	6.68 (d, J=2.0)	94.5	6.89 (d, J=2.1)	93.9
8a	-	157.8	-	157.1
1’	-	123.4	-	125.8
2’	7.39 (d, J=2.0)	110.1	7.35 (s)	104.2
3’	-	149.7	-	153.3
4’	-	153.0	-	140.9
5’	6.96 (d, J=8.5)	112.4	-	153.3
6’	7.53 (dd, J=8.5; J=2.0)	121.0	7.35 (s)	104.2

Table 8 (Contd.)- ^{1}H and ^{13}C NMR data of propargyl flavone derivatives **LuMeProp** and **TriCeProp**.

	LuMeProp		**TriCeProp**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
1”	4.76 (d, J=2.0)	56.9	4.94 (d, J=2.0)	56.7
2”	-	79.7	-	79.1
3”	3.61 (brs)	79.1	3.68 (brs)	78.4
OCH_3	3.69 (s), 3.68 (s)	56.5, 56.4	3.89 (s), 3.74 (s)	56.4, 56.3

Values in parts per million (δ_H and δ_C). Measured in DMSO-d_6 at 300.13 and 75.47 MHz. J values (Hz) are presented in parentheses.

2.2.4. Flavone *O*-acetyl glycosides LuMeTGluAc and TriCeTGluAc

The IR data of *O*-acetyl glycosides **LuMeTGluAc** and **TriCeTGluAc** are in accordance with the predicted structure (**Table 9**). The presence of triazole rings was suggested by the observation of absorption bands at 1351 and 1321 cm^{-1} corresponding to C-N. Additionally, the introduction of *O*-acetyl-*β*-D-glucopyranosyl groups was suggested by the presence of bands at 1757 and 1753 cm^{-1} associated with ester group.

Table 9 – IR data of **LuMeTGluAc** and **TriCeTGluAc**.

Groups	**υ (cm^{-1})**	
	LuMeTGluAc	**TriCeTGluAc**
OH	3600-3300	3600-3300
Aliphatic C-H	2963	2918 2850
C=O		
Ester	1757	1753
Ketone	1655	1659

Table 9 (Contd.)– IR data of **LuMeTGluAc** and **TriCeTGluAc**.

Groups	**υ (cm^{-1})**	
	LuMeTGluAc	**TriCeTGluAc**
Aromatic C=C	1516 1502 1429	1499 1457 1424
C-N	1321	1351
C-O	1258	1226

The ^1H and ^{13}C NMR data of **LuMeTGluAc** and **TriCeTGluAc** are reported in **Table 10**. These spectra showed characteristic signals of the scaffold of the precursors **LuMeProp** and **TriCeProp**. Beyond that, instead of characteristic signals of the propargyl group at C-7, signals of a triazole ring (δ_H 8.60-8.58 s, δ_C 142.7, δ_C 124.0 and δ_C 123.9) and an *O*-acetyl-*β*-glucopyranosyl group are observed (**Figure 9**).

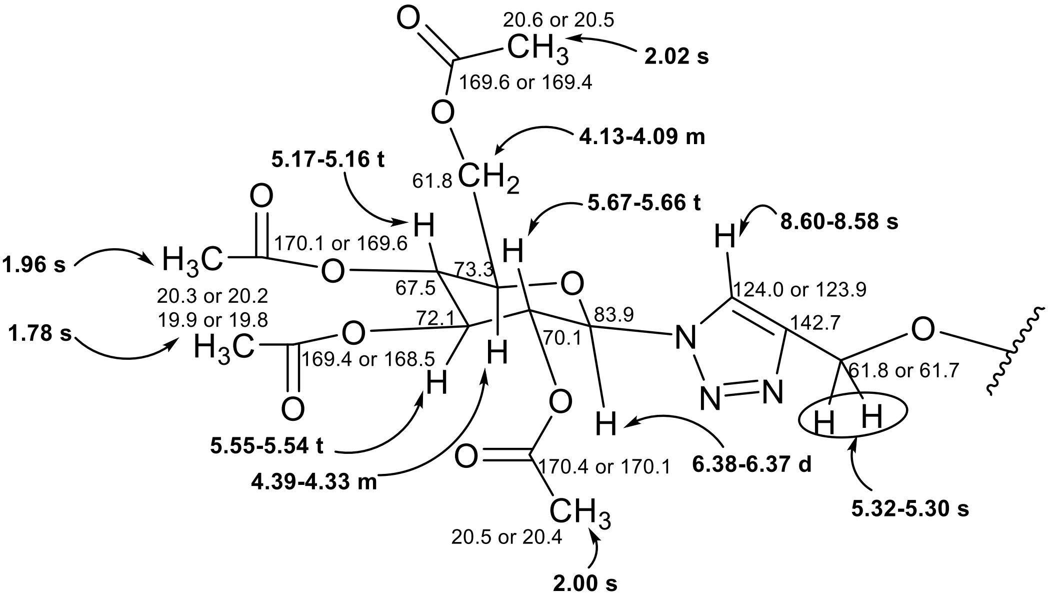


Figure 9- ^1H and ^{13}C NMR data of **LuMeTGluAc** and **TriCeTGluAc**.

The position of this group was evidence by the correlations found in the HMBC indicated in **Figure 10**.

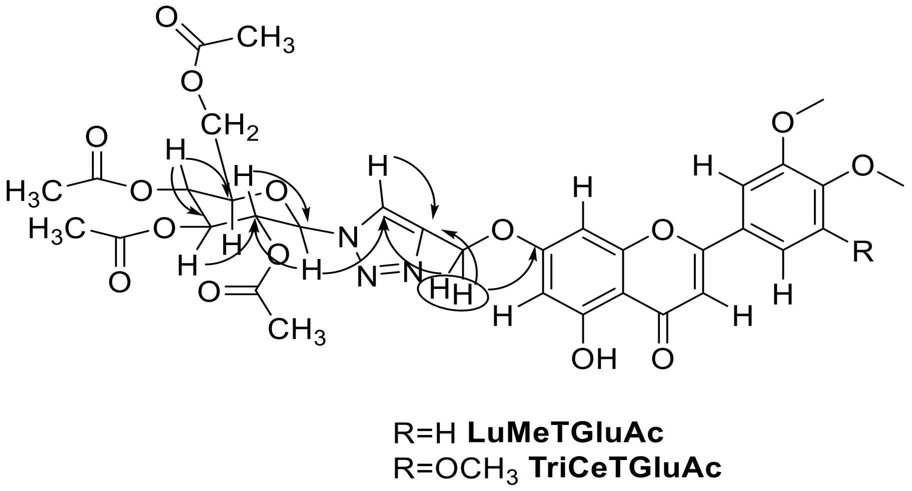


Figure 10– Main connectivities found in the **HMBC of LuMeTGluAc** and **TriCeTGluAc**.

Table 10- ^{1}H and ^{13}C NMR data of **LuMeTGluAc** and **TriCeTGluAc**.

	LuMeTGluAc		**TriCeTGluAc**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
2	-	163.7	-	163.2
3	7.03 (s)	105.0	7.16 (s)	105.1
4	-	182.1	-	182.2
4a	-	104.1	-	105.1
5	12.90 (s, OH)	161.2	12.84 (s, OH)	161.2
6	6.45 (d, J=2.0)	98.7	6.48 (d, J=2.1)	98.8
7	-	168.5	-	163.8
8	6.92 (d, J=2.0)	93.7	6.99 (d, J=2.1)	93.8
8a	-	157.2	-	157.2
1’	-	122.7	-	125.8
2’	7.57 (d, J=2.0)	109.5	7.37 (s)	104.2
3’	-	149.0	-	153.3
4’	-	152.3	-	140.9

Table 10 (Contd.)- ^{1}H and ^{13}C NMR data of **LuMeTGluAc** and **TriCeTGluAc**.

	LuMeTGluAc		**TriCeTGluAc**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
5’	7.12 (d, J=8.5)	111.7	-	153.3
6’	7.69 (dd, J=8.5; J=2.0)	120.2	7.37 (s)	104.2
1”	5.30 (s)	61.7	5.32 (s)	61.8
2”	-	142.7	-	142.7
3”	8.58 (s)	124.0	8.60(s)	123.9
1’’’	6.37 (d, J=9.0)	83.9	6.38 (d, J=9.0)	83.9
2’’’	5.66 (t, J=9.5)	70.1	5.67 (t, J=9.5)	70.1
3’’’	5.54 (t, J=9.5)	72.1	5.55 (t, J=9.5)	72.1
4’’’	5.16 (t, J=9.8)	67.5	5.17 (t, J=9.8)	67.5
5’’’	4.37-4.33 (m)	73.3	4.39-4.35 (m)	73.3
6’’’	4.12-4.09 (m)	61.8	4.13-4.10 (m)	61.8
OCH_3	3.86 (s), 3.83 (s)	55.9, 55.8	3.90 (s), 3.74 (s)	60.3, 56.3
$COCH_3$	2.02 (s), 2.00 (s), 1.96 (s), 1.78 (s)	170.4, 170.1, 169.6, 169.4 ($\underline{C}$=O) 20.5, 20.4, 20.2, 19.8 ($O\underline{C}H_3$)	2.02 (s), 2.00 (s), 1.96 (s), 1.78 (s)	170.1, 169.6, 169.4, 168.5 ($\underline{C}$=O) 20.6, 20.5, 20.3, 19.9 ($O\underline{C}H_3$)

Values in parts per million (δ_H and δ_C). Measured in DMSO-d_6 at 300.13 and 75.47 MHz. J values (Hz) are presented in parentheses.

2.2.5. Flavone *O*-glycosides LuMeTGluOH and TriCeTGluOH

The IR data of *O*-glycosides **LuMeTGluOH** and **TriCeTGluOH** are very similar to those of the precursors **LuMeTGluAc** and **TriCeTGluAc**, except that the bands at 1757 and 1753 cm^{-1} were not observed, suggesting the success of deacetylation of both compounds (**Table 11**).

Table 11 – IR data of **LuMeTGluOH** and **TriCeTGluOH**.

Groups	**υ (cm^{-1})**	
	LuMeTGluOH	**TriCeTGluOH**
OH	3600-3200	3600-3200
Aliphatic C-H	2919	2919 2850
C=O	1664	1662
Aromatic C=C	1518 1500 1457	1593 1504 1463
C-N	1326	1352
C-O	1259	1253

The ^{1}H and ^{13}C NMR data of **LuMeTGluOH** and **TriCeTGluOH** are reported in **Table 12**. These spectra showed characteristic signals of the scaffold of the precursors **LuMeTGluAc** and **TriCeTGluAc**. However, instead of the signals of acetyl groups, characteristic signals of the hydroxylic protons of *O*-*β*-glucopyranosyl group were observed, namely OH-2’”: δ_H 5.15-5.09 d; OH-3’”: δ_H 5.41-5.34 d; OH-4’”: δ_H 4.63-4.57 d; and OH-6’”: δ_H 5.28-5.20 s (**Figure 11**), confirming that the deacetylation was successfully achieved.

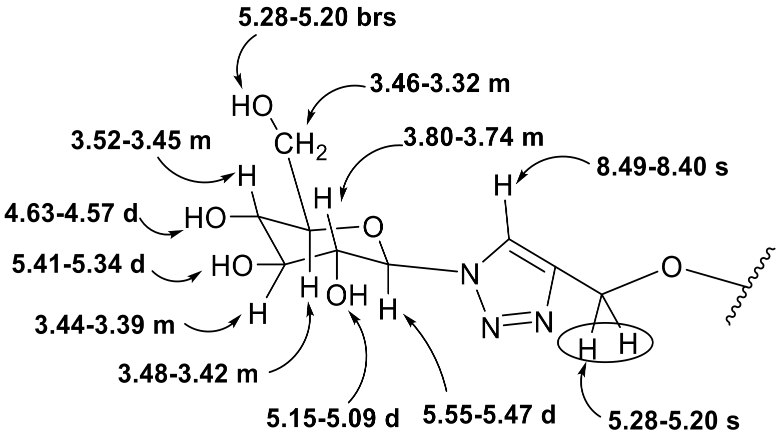


Figure 11 - ^{1}H data of **LuMeTGluOH** and **TriCeTGluOH**.

Table 12 - ^{1}H and ^{13}C NMR data of **LuMeTGluOH** and **TriCeTGluOH**.

	LuMeTGluOH		**TriCeTGluOH**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
2	-	163.7	-	163.5
3	7.03 (s)	105.0	7.05 (s)	105.4
4	-	182.1	-	182.2
4a	-	104.1	-	105.2
5	12.91 (s, OH)	161.2	12.75 (s, OH)	161.2
6	6.47 (d, J=2.1)	98.6	6.41 (d, J=1.8)	98.8
7	-	163.9	-	164.1
8	6.96 (d, J=2.1)	93.5	6.95 (d, J=1.8)	93.8
8a	-	157.3	-	157.4
1’	-	122.8	-	125.9
2’	7.57 (d, J=2.1)	109.5	7.27 (s)	104.3
3’	-	149.0	-	153.4
4’	-	152.3	-	141.0
5’	7.12 (d, J=8.5)	111.7	-	153.4
6’	7.69 (dd, J=8.5; J=2.1)	120.2	7.27 (s)	104.3
1”	5.28 (s)	61.8	5.20 (s)	61.9
2”	-	141.8	-	141.9
3”	8.49 (s)	124.3	8.40 (s)	124.4
1”’	5.55 (d, J=9.3)	87.5	5.47 (d, J=9.0)	87.6
2”’	5.15 (d, J=5.7, OH) 3.80-3.76 (m)	72.0	5.09 (d, J=5.5, OH) 3.78-3.74 (m)	72.1

Table 12 (Contd.) - ^{1}H and ^{13}C NMR data of **LuMeTGluOH** and **TriCeTGluOH**.

	LuMeTGluOH		**TriCeTGluOH**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
3'''	5.41 (d, J=5.7, OH) 3.44-3.40 (m)	77.0	5.34 (d, J=5.5, OH) 3.43-3.39 (m)	77.1
4'''	4.63 (d, J=5.7, OH) 3.52-3.48 (m)	69.6	4.57 (d, J=5.5, OH) 3.50-3.45 (m)	69.9
5'''	3.47-3.44 (m)	80.0	3.48-3.42 (m)	80.1
6'''	5.28 (brs, OH) 3.46-3.42 (m)	60.8	5.20 (brs, OH) 3.38-3.32 (m)	60.8
OCH_3	3.86 (s), 3.83 (s)	55.9, 55.8	3.81 (s), 3.65 (s)	60.4, 56.4

Values in parts per million (δ_H and δ_C). Measured in DMSO-d_6 at 300.13 and 75.47 MHz. J values (Hz) are presented in parentheses.

2.2.6. Flavone *O*-sulfate glycosides LuMeTGluS and TriCeTGluS

The IR data of *O*-sulfate glycosides **LuMeTGluS** and **TriCeTGluS** are presented in **Table 13**. The observation of bands at 1260-1259 cm^{-1} (S=O), 1034-1017 cm^{-1} (C-O-S), and 808-802 cm^{-1} (S-O) suggested the success of sulfation of precursors **LuMeTGluOH** and **TriCeTGluOH.**

Table 13 – IR data of **LuMeTGluS** and **TriCeTGluS**.

Groups	**υ (cm^{-1})**	
	LuMeTGluS	**TriCeTGluS**
OH	3600-3300	3600-3300
Aliphatic C-H	2919	2919
C=O	1660	1617

Table 13 (Contd.)– IR data of **LuMeTGluS** and **TriCeTGluS**.

Groups	**υ (cm^{-1})**	
	LuMeTGluS	**TriCeTGluS**
Aromatic C=C	1517 1503 1455	1501 1465
S=O	1260	1259
C-O-S	1017	1034
S-O	808	802

When compared with the precursors **LuMeTGluOH** and **TriCeTGluOH**, the signals of hydroxyl protons were not observed in the ^{1}H NMR spectra, which confirmed the success of sulfation (**Table 14**). Additionally, the signals characteristic of aliphatic protons (the sugar moiety) were observed between δ_H 5.74-3.65 ppm to **LuMeTGluS** and δ_H 6.23-3.60 ppm to **TriCeTGluS**, whereas the corresponding signals of these protons appeared between δ_H 5.55-3.42 ppm and δ_H 5.47-3.32 ppm in the non-sulfated parent compounds, respectively (**Figure 12**).

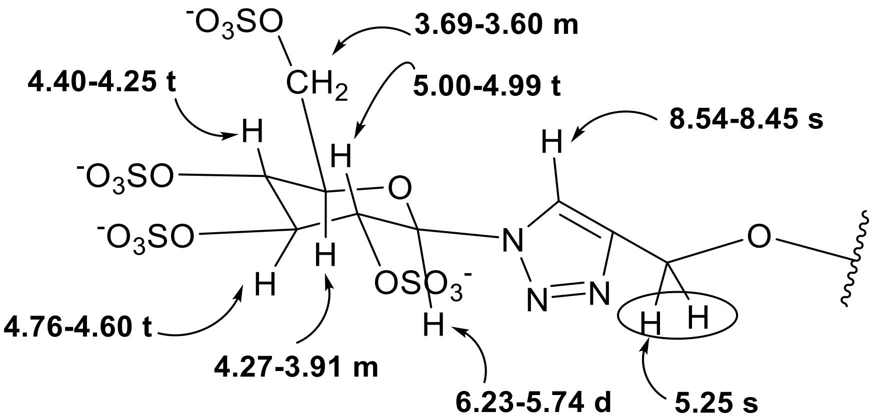


Figure 12 - ^{1}H data of **LuMeTGluS** and **TriCeTGluS**.

Table 14- ^{1}H and ^{13}C NMR data of **LuMeTGluS** and **TriCeTGluS**.

	LuMeTGluS		**TriCeTGluS**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
2	-	161.2	-	163.5
3	6.92 (s)	105.1	7.14 (s)	105.2
4	-	182.2	-	182.3
4a	-	104.1	-	105.2
5	12.81 (s, OH)	157.4	12.84 (s, OH)	161.2
6	6.40 (d, J=2.0)	98.8	6.54 (d, J=2.1)	98.9
7	-	163.8	-	164.2
8	6.90 (d, J=2.0)	93.6	7.10 (d, J=2.1)	93.8
8a	-	152.3	-	157.5
1’	-	122.9	-	126.0
2’	7.49 (d, J=2.0)	109.5	7.40 (s)	104.3
3’	-	142.0	-	153.4
4’	-	149.1	-	140.9
5’	7.04 (d, J=8.0)	111.8	-	153.4
6’	7.62 (dd, J=8.0; J=2.0)	120.3	7.40 (s)	104.3
1”	5.25 (s)	61.9	5.25 (s)	62.0
2”	-	141.4	-	141.9
3”	8.45 (s)	124.3	8.54(s)	124.5
1’’’	5.74 (d, J=9.0)	86.9	6.23 (d, J=9.3)	87.5
2’’’	5.00 (t, J=3.5)	70.8	4.99 (t, J=3.6)	71.7
3’’’	4.60 (t, J=3.3)	77.1	4.76 (t, J=3.5)	78.7
4’’’	4.40 (t, J=2.5)	68.7	4.25 (t, J=3.0)	69.0
5’’’	4.27-4.23 (m)	82.4	3.96-3.91 (m)	83.9

Table 14 (Contd.) - ^{1}H and ^{13}C NMR data of **LuMeTGluS** and **TriCeTGluS**.

	LuMeTGluS		**TriCeTGluS**	
	^{1}H	**^{13}C**	**^{1}H**	**^{13}C**
6’’’	3.69-3.65 (m)	65.6	3.65-3.60 (m)	66.6
OCH_3	3.77 (s), 3.74 (s)	56.0, 55.8	3.82 (s), 3.76 (s)	60.4, 56.5

Values in parts per million (δ_H and δ_C). Measured in DMSO-d_6 at 500.16 and 125.77 MHz. J values (Hz) are presented in parentheses.

2.3. Antifouling Activity

As biofouling results from the adhesion and growth of marine biofouling organisms, including microfouling (e.g. bacteria, diatoms, and algal spores) and macrofouling organisms (e.g. barnacle larvae), a suitable strategy for the implementation of an effective antifouling screening should include the evaluation of the anti-settlement activity against macrofouling and microfouling species. In this research project preliminary studies concerning the screening on antifouling activity against macrofoulers were developed using *in vivo* assays. Considering that *Mytilus* spp. are one of the most common biofouling species and that the larval phases responsible for the initial settlement of *Mytilus galloprovincialis* have produced consistent results in anti-settlement bioassays,[67-71] the potential of all synthesized compounds were evaluated using the anti-settlement activity of mussel (*Mytilus galloprovincialis*) plantigrade post-larvae assay. In addition, other structure related flavonoids with a chalcone scaffold, previously synthesized in LQOF/CIIMAR research group (**Figure 13**), were evaluated in order to propose some SAR considerations.

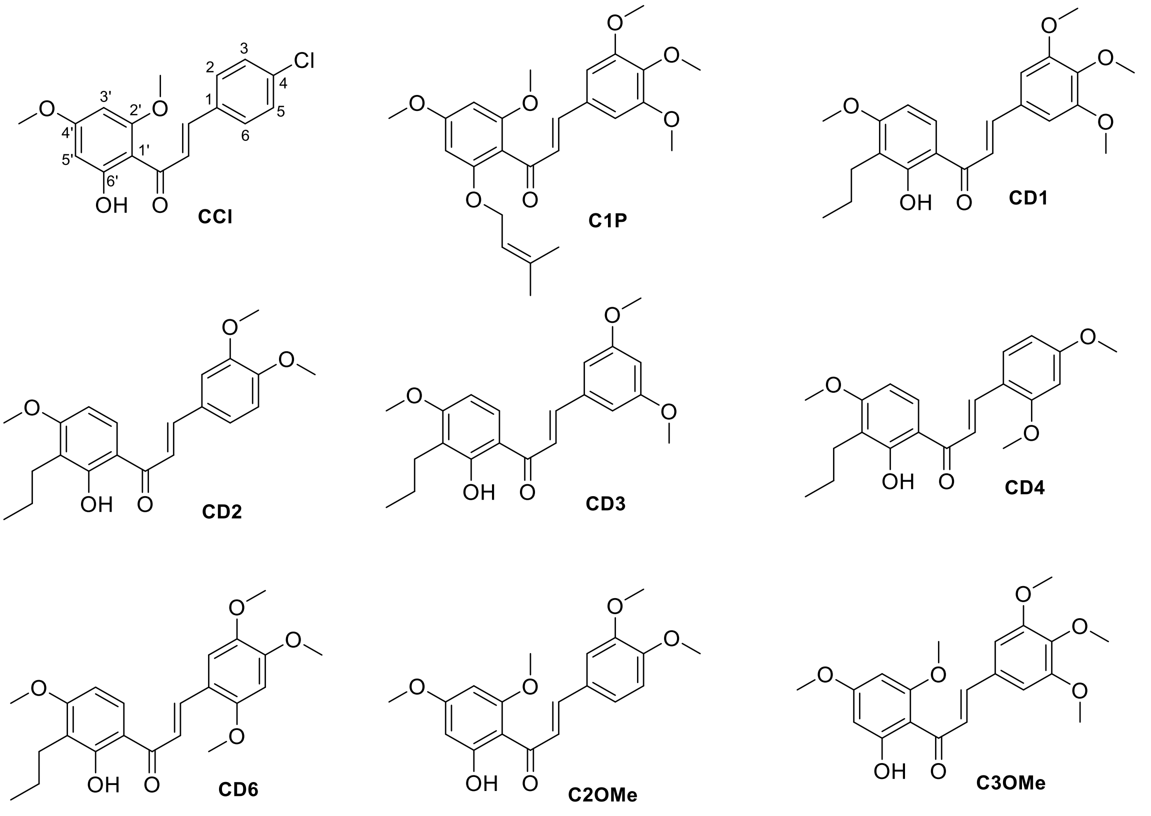


Figure 13 - Structure of chalcones previously synthesized in LQOF/CIIMAR research group and tested for antifouling activity.

Figure 14 represents the percentage of settlement of *M. galloprovincialis* after treatment of mussel with 50 µM of each compound.

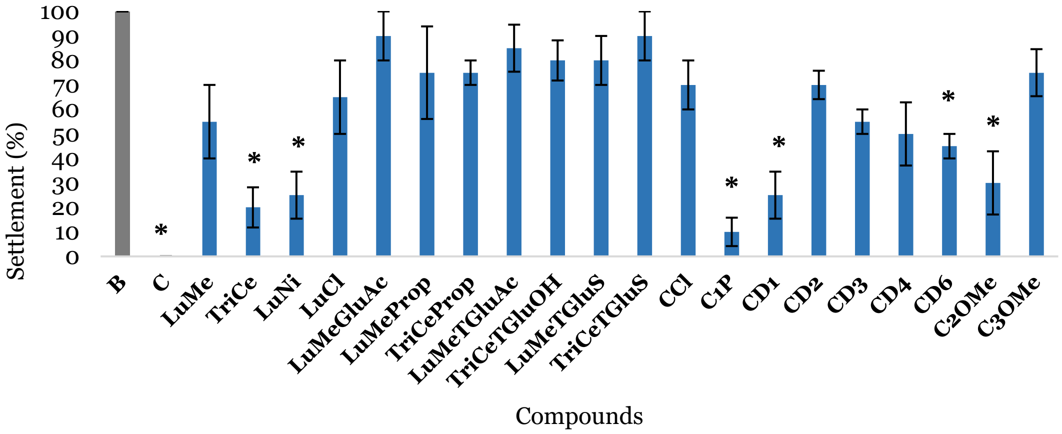


Figure 14 – Anti-settlement activity of compounds towards plantigrades of the mussel *Mytilus galloprovincialis*. *Indicates significant differences (p<0.05) against the negative control (**B**: ultra-pure water); CuSO4 at 5 µM was used as positive control (**C**).

From the series of studied derivatives, six compounds (**TriCe**, **LuNi**, **C1P**, **CD1**, **CD6**, and **C2OMe**) showed a significant inhibitory effect (*p*<0.05) against the settlement of *M. galloprovincialis* plantigrades, and fourteen compounds were not significantly different from the negative control (**Figure 14**). In the future a series of concentrations should be tested in order to determine EC_{50} and LD_{50}. Additionally, the results should be compared with one commercial eco-friendly antifouling agent, tested in the same experimental conditions, to obtain comparable data regarding effectiveness and toxicity of compounds.

Although the number of compounds was limited, an attempt was made to draw some considerations concerning SAR.

Considering the synthesized flavones, the most promising results were obtained for **TriCe** and **LuNi**, possessing 3,4,5-trimethoxy- and 4-nitro- phenyl B rings, respectively. In contrast, all synthesized **LuMe** and **TriCe** 7-*O*-glycosides showed a percentage of settlement similar to the negative control, suggesting the importance of the hydroxyl group at C-7 for this activity. Nevertheless, this result should be confirmed after the synthesis and evaluation of other structure related flavone glycosides using **LuNi** and **LuCl** as building blocks.

Considering chalcones some interesting results were observed. Comparing results for chalcones **CD1-CD6,** it seems that the presence of a 3,4,5-trimethoxyphenyl B ring is associated with a high anti-settlement activity. In fact, among these chalcones, **CD1** with three methoxyl groups at positions 3,4,5 showed the best ativity, suggesting the importance of this substitution pattern on B ring. Nevertheless, the comparison of activity of **C1P**, **CD1**, and **C3OMe** revealed that in addition to the presence of a 3,4,5-trimethoxy phenyl B ring the presence of an alkyl side chain in A ring seems also to be associated with a more promissing anti-settlement activity. In fact, compounds **CD1** and **C1P** with a 3-propyl or a 2-oxyprenyl group, respectively, revealed a better activity than **C3OMe.**

2.4 The environmental fate predictions

Due to the toxicity of biocides, environmental legislation for the registration of chemicals for use in the environment has grown to a monumental challenge requiring product dossiers to include information on the environmental fate and behavior of any chemicals. So, persistence, bioaccumulation, and toxicity, known as PBT, are key traits to be monitored for compounds and their degradation products.[72] Nevertheless, experimental tecniques of PBT properties evaluation are expensive process, and thus not applied in the early stages of the product discovery and development. Therefore, *in silico* methods for preliminary PBT screening should be applied.

Due to this, we evaluated the PBT properties of compounds, which appear to have promising antifouling activity, using prediction programs from the Environmental Protection Agency (EPA), commonly used in the registration process of biocides.

The bioaccumulation was estimated using the software log octanol-water partition coefficient (log K_{ow}) calculation program KOWWIN™. Compounds are considered potentially bioaccumulative if the log K_{ow} is 3 or more.[72] Water solubility was predicted using WSKOWWIN™. This program estimates the water solubility of an organic compound using the log K_{ow} of compounds.

The biodegradation was estimated using the Biodegradation Probability Program BIOWIN™. This software utilizes functional group contribution approaches in the estimation of aerobic biodegradation of organic compounds in the presence of microorganisms in the environment. Within BIOWIN™ seven separate models (BIOWIN™ 1-7) can be used. Different researchers have used the predictions from a combination of different models to estimate biodegradability.[72] In practice, combining models has been shown to improve the accuracy of predictions.[72] For a compound to be considered as biodegradable it must obay to one of the following criteria: Criteria A - BIOWIN™ 2 or 6 > 0.5, and BIOWIN™ 3 > 2.2; Criteria B - BIOWIN™ 3 “weeks” or faster, and BIOWIN™ 5 > 0.5. The BIOWIN™ program automatically generates a yes/no qualitative prediction of ready biodegradability.

In addition to most promising antifouling compounds (**TriCe**, **LuNi**, **C1P**, **CD1**, **CD6,** and **C2OMe**), *in silico* studies were also performed for a series of commercial biocides (irgarol 1051, dicholofuanid, and chlorothalonil),[72] for comparison purposes (**Table 15**).

Table 15 - Results of biodegradation and bioaccumulation predictions using BIOWIN™ and KOWWIN™ softwares.

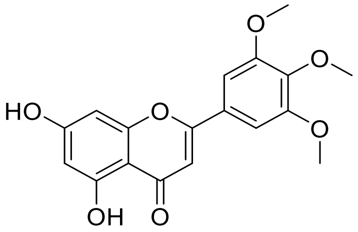
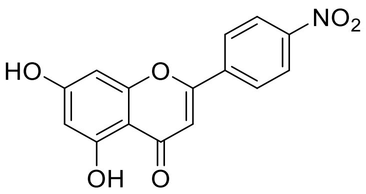
Name	***M*$_r$**	**Water solubility at 25 °C (mg/L)**	**Log K$_{ow}$**	**BIOWIN™**
 **TriCe**	344.32	96.52	2.84	BIOWIN™ 1: 1.35 BIOWIN™ 2: 1.00 BIOWIN™ 3: 2.30 BIOWIN™ 4: 3.72 BIOWIN™ 5: 0.76 BIOWIN™ 6: 0.56 BIOWIN™ 7: 0.62 Criteria A: YES Criteria B: NO
 **LuNi**	299.24	39.80	3.14	BIOWIN™ 1: 0.67 BIOWIN™ 2: 0.47 BIOWIN™ 3: 2.40 BIOWIN™ 4: 3.44 BIOWIN™ 5: 0.13 BIOWIN™ 6: 0.01 BIOWIN™ 7: -0.08 Criteria A: NO Criteria B: NO

Table 15 (Contd.) - Results of biodegradation and bioaccumulation predictions using BIOWIN™ and KOWWIN™ softwares.

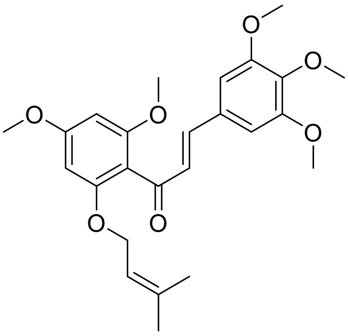
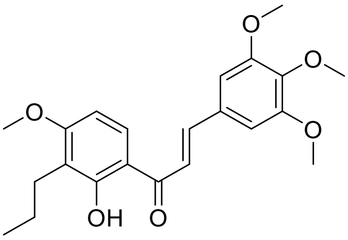
Name	***M*_r**	**Water solubility at 25°C (mg/L)**	**Log K_{ow}**	**BIOWIN™**
 **C1P**	442.51	0.06	5.22	BIOWIN™ 1: 1.34 BIOWIN™ 2: 1.00 BIOWIN™ 3: 1.85 BIOWIN™ 4: 3.65 BIOWIN™ 5: 0.79 BIOWIN™ 6: 0.41 BIOWIN™ 7: 0.38 Criteria A: NO Criteria B: NO
 **CD1**	386.45	0.65	5.08	BIOWIN™ 1: 1.27 BIOWIN™ 2: 1.00 BIOWIN™ 3: 2.07 BIOWIN™ 4: 3.55 BIOWIN™ 5: 0.57 BIOWIN™ 6: 0.25 BIOWIN™ 7: 0.21 Criteria A: NO Criteria B: NO

Table 15 (Contd.) - Results of biodegradation and bioaccumulation predictions using BIOWIN™ and KOWWIN™ softwares.

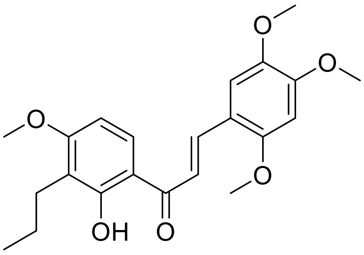
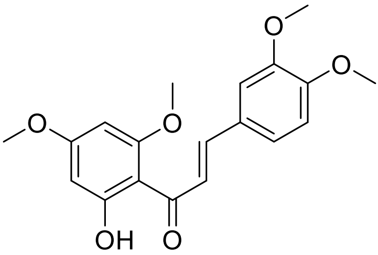
Name	***M*$_r$**	**Water solubility at 25°C (mg/L)**	**Log K_{ow}**	**BIOWIN™**
 **CD6**	386.45	0.43	5.29	BIOWIN™ 1: 1.27 BIOWIN™ 2: 1.00 BIOWIN™ 3: 2.07 BIOWIN™ 4: 3.55 BIOWIN™ 5: 0.57 BIOWIN™ 6: 0.25 BIOWIN™ 7: 0.21 Criteria A: NO Criteria B: NO
 **C2OMe**	344.37	15.63	3.76	BIOWIN™ 1: 1.23 BIOWIN™ 2: 1.00 BIOWIN™ 3: 2.24 BIOWIN™ 4: 3.68 BIOWIN™ 5: 0.71 BIOWIN™ 6: 0.48 BIOWIN™ 7: 0.29 Criteria A: NO Criteria B: NO

Table 15 (Contd.) - Results of biodegradation and bioaccumulation predictions using BIOWIN™ and KOWWIN™ softwares.

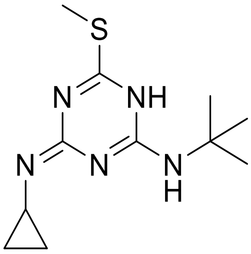
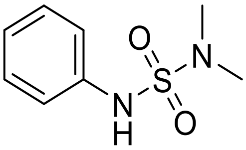
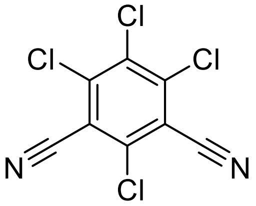
Name	M_r	Water solubility at 25°C (mg/L)	Log K_{ow}	BIOWIN™
 **Irgarol 1051**	253.37	7.52	4.07	BIOWIN™ 1: 0.44 BIOWIN™ 2: 0.09 BIOWIN™ 3: 2.43 BIOWIN™ 4: 3.33 BIOWIN™ 5: 0.08 BIOWIN™ 6: 0.02 BIOWIN™ 7: -0.02 Criteria A: NO Criteria B: NO
 **Dicholofuanid**	333.22	5.41	3.72	BIOWIN™ 1: 0.31 BIOWIN™ 2: 0.01 BIOWIN™ 3: 1.93 BIOWIN™ 4: 3.02 BIOWIN™ 5: -0.15 BIOWIN™ 6: 0.00 BIOWIN™ 7: 0.05 Criteria A: NO Criteria B: NO

Table 15 (Contd.) - Results of biodegradation and bioaccumulation predictions using BIOWIN™ and KOWWIN™ softwares.

Name	***M*$_r$**	**Water solubility at 25°C (mg/L)**	**Log K_{ow}**	**BIOWIN™**
 **Chlorothalonil**	265.91	26.01	3.66	BIOWIN™ 1: 0.51 BIOWIN™ 2: 0.61 BIOWIN™ 3: 1.62 BIOWIN™ 4: 2.67 BIOWIN™ 5: 0.09 BIOWIN™ 6: 0.01 BIOWIN™ 7: -0.77 Criteria A: NO Criteria B: NO

After analysis of the KOWWIN™ program, in contrast to the tested commercial biocides, **TriCe** has presented a favourable result showing low bioaccumulative potential, revealing a log K_{ow} lower than 3.

Considering BIOWIN™ results, among the tested compounds **TriCe** is the only one that is readily biodegradable under criteria A. Under criteria B, none of the tested compounds are predicted to be readily biodegradable, including the commercial biocides.

Adding to the low potential for bioaccumulation and high biodegradability, the relative high water solubility of **TriCe** allows to predict the potential of this compound as "environmentally-benign" antifoulant and the synthesis of **TriCe** analogues should be explored in the future.

CHAPTER III

Conclusions and Perspectives

The investigation of marine world is growing due to the fact that it is one of the largest biological resources, containing a vast array of organisms with unique biological systems and characteristics that are important sources of bioactive natural products with pharmaceutical and chemical applications. Among these natural products, some marine flavonoids, including sulfated glycosylated flavones, such as thalassiolin A, with promising antifouling activities have been isolated.

The current work allowed the synthesis of thirteen flavones, including three flavone glycosides structure related with thalassiolin A. On the basis of our current state of knowledge nine of these flavones are described for the first time. The total synthesis of sulfated glycosylated flavone derivatives were successfully accomplished using two building blocks, **LuMe** and **TriCe**.

In addition, the potential of all synthetized flavones, as well as structure related chalcones, as antifouling agents against macrofouling species was screened by the anti-settlement activity of mussel (*Mytilus galloprovincialis*) plantigrade post-larvae.

From the preliminary antifouling screening, six compounds (**TriCe**, **LuNi**, **C1P**, **CD1**, **CD6**, and **C2OMe**) were identified as promising antifouling agents against macrofouling species. Nevertheless, in the future for these compounds a series of concentrations should be tested in order to determine EC_{50} and LD_{50}.

These results combined with *in silico* studies concerning the prediction of biodegradability and bioaccumulation in aquatic environment suggest the potential of **TriCe** as a new "environmentally-benign" antifoulant. Following this, the synthesis of **TriCe** analogues should be explored in the future.

Additionally, the effect against microfouling species of all synthesized compounds, as well as other sulfated glycosylated flavones using **LuNi** and **LuCl** as building blocks, should be studied in the future. In fact, although the sulfated glycosylated flavones with a triazole ring (**LuMeTGluS** and **TriCeTGluS**) did not reveal significant anti-settlement activity against macrofoulers, as triazole rich molecules have been described for their inhibitory effects on the settlement of microfouling species,[59] the anti-settlement activity against these microorganisms deserve to be explore in the future.

CHAPTER IV

Experimental Procedures

4.1. General Methods

MW reactions were performed using a glassware setup for atmospheric-pressure reactions and a 100 mL or 30 mL teflon reactor (internal reaction temperature was controlled by a fiber-optic probe sensor) and were performed using an Ethos MicroSYNTH 1600 Microwave Labstation from Milestone (ThermoUnicam, Portugal).

All reactions were controlled by TLC using Merck silica gel 60 (GF254) plates with 0.2 mm of thickness. The visualization of the chromatograms was under UV light at 254 and 365 nm.

Purification of the synthesized compounds was carried out by flash column chromatography using Macherey-Nagel silica gel 60 (0.04-0.063 mm), preparative TLC using Macherey-Nagel silica gel 60 (GF254) plates, and crystallization.

Melting points were obtained in a Köfler microscope and are uncorrected.

IR spectra were obtained in KBr microplates in a Fourier transform infrared spectroscopy (FTIR) spectrometer Nicolet iS10 from Thermo Scientific with Smart OMNI-Transmisson accessory (Software OMNIC 8.3) (cm^{-1}).

^1H and ^{13}C NMR spectra were performed in University of Aveiro, Department of Chemistry and were taken in $CDCl_3$ and DMSO-d_6 (Deutero GmbH), at room temperature, on Bruker Avance 300 instrument (300.13 MHz for ^1H and 75.47 MHz for ^{13}C) and 500 instrument (500.16 MHz for ^1H and 125.77 MHz for ^{13}C). ^{13}C NMR assignments were made by 2D HSQC and HMBC experiments (long-range C, H coupling constants were optimized to 7 Hz). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz).

All commercially available reagents were purchased from Sigma Aldrich Co. Reagents and solvents were purified and dried according to the usual procedures described elsewhere.[73] The following materials were synthesized and purified by the described procedures.

4.2. Synthesis

4.2.1. Synthesis of flavone derivatives LuMe and TriCe

A mixture of phloroglucinol (175 mg, 1 mmol) and ethyl 3,4-dimethoxybenzoylacetate/ ethyl 3,4,5-trimethoxybenzoylacetate (700/782 mg, 2 mmol) was heated at 240 °C in muffle furnace for 60-100 min. The crude mixture was dissolved in 10% aq NaOH (20 mL) and washed with diethyl ether (2x20 mL), and the product was precipitated by adding 37% aq HCl. The solid was filtered, washed with water, and vacuum-dried.

Luteolin 3’,4’-dimethyl ether (LuMe). Yield: 74%; mp 280-281 ºC; IR (kBr) υmax: 3600-3300, 2950, 1655, 1581, 1517, 1504, 1429, 1256 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 300.13 MHz), δ: 12.92 (1H, s, OH-5), 10.97 (1H, s, OH-7), 7.68 (1H, dd, J = 8.7 and 2.1 Hz, H-6’), 7.56 (1H, d, J = 2.1 Hz, H-2’), 7.12 (1H, d, J = 8.7 Hz, H-5’), 6.96 (1H, s, H-3), 6.55 (1H, d, J = 2.1 Hz, H-8), 6.23 (1H, d, J = 2.1 Hz, H-6), 3.88 (3H, s, OCH$_3$, H-4’), 3.85 (3H, s, OCH$_3$, H-3’). ^{13}C NMR (DMSO-d$_6$, 75.47 MHz) δ: 181.9 (C4), 164.3 (C7), 163.4 (C2), 161.4 (C5), 157.4 (C8a), 152.2 (C4’), 149.0 (C3’), 122.9 (C1’), 120.1 (C6’), 111.7 (C5’), 109.4 (C2’), 103.9 (C3), 103.8 (C4a), 99.0 (C6), 94.2 (C8), 55.9 (OCH$_3$), 55.8 (OCH$_3$).

Tricetin trimethyl ether (TriCe). Yield: 77%; mp 268-270 ºC; IR (kBr) υmax: 3600-3300, 2950, 2839, 1655, 1592, 1558, 1505, 1441, 1257 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 300.13 MHz), δ: 12.86 (1H, s, OH-5), 10.94 (1H, s, OH-7), 7.33 (2H, s, H-2’ and H-6’), 7.07 (1H, s, H-3), 6.59 (1H, d, J = 2.1 Hz, H-8), 6.23 (1H, d, J = 2.1 Hz, H-6), 3.90 (6H, s, OCH$_3$, H-3’and H-5’), 3.75 (3H, s, OCH$_3$, H-4’). ^{13}C NMR (DMSO-d$_6$, 75.47 MHz) δ: 182.0 (C4), 164.4 (C7), 163.1 (C2), 161.4 (C5), 157.5 (C8a), 153.3 (C3’ and 5’), 140.8 (C4’), 126.0 (C1’), 105.1 (C3), 104.1 (C2’ and 6’), 103.9 (C4a), 99.0 (C6), 94.4 (C8), 60.3 (OCH$_3$), 56.3 (OCH$_3$).

4.2.2. Synthesis of flavone derivatives LuNi and LuCl

A mixture of phloroglucinol (175 mg, 1 mmol) and ethyl 4-chlorobenzoylacetate/ ethyl 4-nitrobenzoylacetate (628/782 mg, 2 mmol) was heated at 240 °C in muffle furnace for 50-80 min. The crude mixture was dissolved in 10% aq NaOH (20 mL) and washed with diethyl ether (2x20 mL), and the product was precipitated by adding 37% aq HCl. The solid was filtered, washed with water, vacuum-dried, and purified by flash column chromatography (SiO_2; n-hexane: EtOAc, 8:2) followed by crystallization in acetone.

5,7-Dihydroxy-4'-nitroflavone (LuNi). Yield: 16%; mp 284-285 °C; IR (kBr) υmax: 3600-3300, 1653, 1598, 1518, 1456, 1431, 1372, 1274 cm^{-1}; ^1H NMR (DMSO-d_6, 300.13 MHz), δ: 12.53 (1H, s, OH-5), 10.54 (1H, s, OH-7), 8.38 (2H, d, J = 8.9 Hz, H-3' and H-5'), 8.16 (2H, d, J = 8.9 Hz, H-2' and H-6'), 6.86 (1H, s, H-3), 6.49 (1H, d, J = 2.0 Hz, H-8), 6.29 (1H, d, J = 2.0 Hz, H-6). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 181.3 (C4), 164.6 (C7), 161.5 (C2), 160.3 (C5), 157.3 (C8a), 148.8 (C4'), 136.7 (C1'), 127.1 (C3' and 5'), 123.7 (C2' and 6'), 107.4 (C3), 104.2 (C4a), 99.3 (C6), 94.0 (C8).

5,7-Dihydroxy-4'-chloroflavone (LuCl). Yield: 21%; mp 297-299 °C; IR (kBr) υmax: 3600-3300, 1655, 1586, 1575, 1510, 1493, 1269, 785 cm^{-1}; ^1H NMR (DMSO-d_6, 300.13 MHz), δ: 12.77 (1H, s, OH-5), 8.09 (2H, d, J = 9.2 Hz, H-2' and H-6'), 7.63 (2H, d, J = 9.2 Hz, H-3' and H-5'), 7.00 (1H, s, H-3), 6.52 (1H, d, J = 2.1 Hz, H-8), 6.22 (1H, d, J = 2.1 Hz, H-6). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 181.8 (C4), 164.5 (C7), 162.0 (C2), 161.5(C5), 157.4 (C8a), 136.8 (C4'), 129.6 (C1'), 129.2 (C2' and 6'), 128.3 (C3' and 5'), 105.5 (C3), 104.0 (C4a), 99.1 (C6), 94.2 (C8).

4.2.3. Synthesis of flavone *O*-acetyl glycoside LuMeGluAc

A suspension of **LuMe** (200 mg, 0.64 mmol), acetobromo-α-D-glucose (288 mg, 0.70 mmol), silver carbonate (Ag_2CO_3) (193 mg, 0.70 mmol), and ground 3 Å molecular sieves in dichloromethane (CH_2Cl_2) (20 mL) was stirred under nitrogen at room temperature. The mixture was stirred in the dark for 7 days, and filtered through celite. The latter was washed with 10% MeOH-CH_2Cl_2 (50 mL). The filtrates were combined and washed successively with 10% aq acetic acid (AcOH) (2x50 mL), water (1x100 mL), 0.1 M sodium thiosulfate ($Na_2S_2O_3$) (1x100 mL), water (1x100 mL), satd sodium bicarbonate ($NaHCO_3$) (1x100 mL), and water (1x100 mL), and dried with sodium sulfate (Na_2SO_4). After drying, by evaporation, the solid was dissolved in 50 mL 1% MeOH-CH_2Cl_2 and cooled to 0 °C overnight. After filtration, the precipitated **LuMe** was removed and the filtrate was evaporated. Impurities and further residual **LuMe** were removed through flash column chromatography (SiO_2; n-hexane: EtOAc, 8:2) followed by crystallization in acetone.

7-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)luteolin 3’,4’-dimethyl ether (LuMeGluAc). Yield: 7%; mp 219-220 °C; IR (KBr) υmax: 3600-3300, 2963, 1746, 1647, 1603, 1518, 1505, 1456, 1261 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300.13 MHz), δ: 12.81 (1H, s, OH-5), 7.53 (1H, dd, J = 8.7 and 2.1 Hz, H-6’), 7.34 (1H, d, J = 2.1 Hz, H-2’), 6.99 (1H, d, J = 8.7 Hz, H-5’), 6.60 (1H, s, H-3), 6.58 (1H, d, J = 2.1 Hz, H-8), 6.45 (1H, d, J = 2.1 Hz, H-6), 4.98 (1H, d, J = 9.0 Hz, H-1”), 4.77-3.97 (6H, m, H-2”-6”), 3.99 (3H, s, OCH_3, H-4’), 3.98 (3H, s, OCH_3, H-3’), 2.19 (3H, s, $COCH_3$), 2.08 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.00 (3H, s, $COCH_3$). ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ: 182.4 (C4), 170.6 ($\underline{C}OCH_3$), 170.1 ($\underline{C}OCH_3$), 169.5 ($\underline{C}OCH_3$), 169.2 ($\underline{C}OCH_3$), 164.6 (C7), 164.2 (C2), 163.2 (C5), 163.0 (C8a), 162.2 (C4’), 157.6 (C3’), 123.7 (C1’), 120.2 (C6’), 111.2 (C5’), 108.8 (C2’), 104.8 (C3), 104.3 (C4a), 98.8 (C6), 97.7 (C1”), 93.6 (C8), 77.2 (C2”-6”), 76.9 (C2”-6”), 76.5 (C2”-6”), 74.2 (C2”-6”), 73.2 (C2”-6”), 56.2 (OCH_3), 56.1 (OCH_3), 20.7 ($CO\underline{C}H_3$), 20.6 ($CO\underline{C}H_3$), 20.1 ($CO\underline{C}H_3$), 19.8 ($CO\underline{C}H_3$).

4.2.4. Synthesis of propargyl flavone derivatives LuMeProp and TriCeProp

To a solution of **LuMe/TriCe** (200 mg, 0.64/0.58 mmol), cesium carbonate (Cs_2CO_3) (FLUKA 20959, 209 mg, 1 eq.), and tetrabutylammonium bromide (TBAB) (SIGMA-ALDRICH 86868, 206 mg, 1 eq.) in anhydrous acetone (20 mL), propargyl bromide solution (SIGMA-ALDRICH P51001, 80% wt% in toluene, 0.9 mL, 1 eq.) was added. The mixture was refluxed at 60 °C during 6 hours and filtered. The filtrate was evaporated and purification was carried out by flash column chromatography (SiO_2; n-hexane:EtOAc, 8:2) followed by crystallization in acetone.

7-(prop-2-yn-yloxy)luteolin 3’,4’-dimethyl ether (LuMeProp). Yield: 66%; mp 223 °C; IR (KBr) υmax: 3600-3300, 2963, 2917, 2849, 2360, 1647, 1518, 1504, 1468, 1453, 1429, 1261 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300.13 MHz), δ: 12.74 (1H, s, OH-5), 7.53 (1H, dd, J = 8.5 and 2.0 Hz, H-6’), 7.39 (1H, d, J = 2.0 Hz, H-2’), 6.96 (1H, d, J = 8.5 Hz, H-5’), 6.84 (1H, s, H-3), 6.68 (1H, d, J = 2.0 Hz, H-8), 6.26 (1H, d, J = 2.0 Hz, H-6), 4.76 (2H, d, J = 2.0 Hz, H-1”), 3.69 (3H, s, OCH_3, H-4’), 3.68 (3H, s, OCH_3, H-3’), 3.61 (1H, brs, H-3”). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.8 (C4), 164.5 (C7), 163.7 (C2), 161.8 (C5), 157.8 (C8a), 153.0 (C4’), 149.7 (C3’), 123.4 (C1’), 121.0 (C6’), 112.4 (C5’), 110.1 (C2’), 105.8 (C3), 104.8 (C4a), 99.3 (C6), 94.5 (C8), 79.7 (C2”), 79.1 (C3”), 56.9 (C1”), 56.5 (OCH_3), 56.4 (OCH_3).

7-(prop-2-yn-yloxy)tricetin trimethyl ether (TriCeProp). Yield: 55%; mp 201 °C; IR (kBr) υmax: 3600-3300, 2919, 2360, 1647, 1596, 1497, 1456, 1423, 1258 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300.13 MHz), δ: 12.85 (1H, s, OH-5), 7.35 (2H, s, H-2’ and H-6’), 7.15 (1H, s, H-3), 6.89 (1H, d, J = 2.1 Hz, H-8), 6.44 (1H, d, J = 2.1 Hz, H-6), 4.94 (2H, d, J = 2.0 Hz, H-1”), 3.89 (6H, s, OCH_3, H-3’ and H-5’), 3.74 (3H, s, OCH_3, H-4’), 3.68 (1H, brs, H-3”). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.2 (C4), 163.4 (C7), 163.1 (C2), 161.2 (C5), 157.1 (C8a), 153.3 (C3’ and C5’), 140.9 (C4’), 125.8 (C1’), 105.3 (C3 and C4a), 104.2 (C2’ and C6’), 98.7 (C6), 93.9 (C8), 79.1 (C2”), 78.4 (C3”), 56.7 (C1”). 56.4 (OCH_3), 56.3 (OCH_3).

4.2.5. Synthesis of flavone *O*-acetyl glycosides LuMeTGluAc and TriCeTGluAc

To a solution of **LuMeProp/TriCeProp** (100 mg, 0.28/0.26 mmol) and tetraacetate *β*-glucopyranosyl azide (105 mg, 0.28 mmol; 97 mg, 0.26 mmol) in tetrahydrofuran (THF)/water (H_2O) solvent mixture (2:1; 30 mL), sodium ascorbate ($C_6H_7NaO_6$) (Sigma A 7631, 222 mg, 1.12 mmol; 206 mg, 1.04 mmol) and copper(II) sulfate pentahydrate ($Cu_2SO_4.5H_2O$) (Panreac 131270, 140 mg, 0.56 mmol; 130 mg, 0.52 mmol) were added. The reaction vessel was sealed and the mixture was kept stirring and heated for 30 minutes at 70 °C under MW irradiation of 500 W (2 cycles: 5 minutes to achieve 70 °C and 10 minutes under 70 °C). After cooling, the reaction mixture was filtered. After concentration under reduced pressure, the water suspension was extracted twice with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, concentrated under reduced pressure, and then purified by crystallization in acetone.

7-((2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)luteolin 3’,4’-dimethyl ether (LuMeTGluAc). Yield: 82%; mp 155 °C; IR (kBr) υmax: 3600-3300, 2963, 1757, 1655, 1516, 1502, 1429, 1321, 1258 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300.13 MHz), δ: 12.90 (1H, s, OH-5), 8.58 (1H, s, H-3”), 7.69 (1H, dd, J = 8.5 and 2.0 Hz, H-6’), 7.57 (1H, d, J = 2.0 Hz, H-2’), 7.12 (1H, d, J = 8.5 Hz, H-5’), 7.03 (1H, s, H-3), 6.92 (1H, d, J = 2.0 Hz, H-8), 6.45 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 9.0 Hz, H-1’”), 5.66 (1H, t, J = 9.5 Hz, H-2’”), 5.54 (1H, t, J = 9.5 Hz, H-3’”), 5.30 (2H, s, H-1”), 5.16

(1H, t, J = 9.8 Hz, H-4’”), 4.37-4.33 (1H, m, H-5’”), 4.12-4.09 (2H, m, H-6’”), 3.86 (3H, s, OCH_3, H-4’), 3.83 (3H, s, OCH_3, H-3’), 2.02 (3H, s, $COCH_3$), 2.00 (3H, s, $COCH_3$), 1.96 (3H, s, $COCH_3$), 1.78 (3H, s, $COCH_3$). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.1 (C4), 170.4 ($\underline{C}OCH_3$), 170.1 ($\underline{C}OCH_3$), 169.9 ($\underline{C}OCH_3$), 169.4 ($\underline{C}OCH_3$), 168.5 (C7), 163.7 (C2), 161.2 (C5), 157.2 (C8a), 152.3 (C4’), 149.0 (C3’), 142.7 (C2”), 124.0 (C3”), 122.7 (C1’), 120.2 (C6’), 111.7 (C5’), 109.5 (C2’), 105.0 (C3), 104.1 (C4a), 98.7 (C6), 93.7 (C8), 83.9 (C1’”), 73.3 (C5’”), 72.1 (C3’”), 70.1 (C2’”), 67.5 (C4’”), 61.8 (C6’”), 61.7 (C1”), 55.9 (OCH_3), 55.8 (OCH_3), 20.5 ($CO\underline{C}H_3$), 20.4 ($CO\underline{C}H_3$), 20.2 ($CO\underline{C}H_3$), 19.8 ($CO\underline{C}H_3$).

7-((2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)tricetin trimethyl ether (TriCeTGluAc). Yield: 79%; mp 132 ºC; IR (kBr) υmax: 3600-3300, 2918, 2850, 1753, 1659, 1499, 1457, 1424, 1351, 1226 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300.13 MHz), δ: 12.84 (1H, s, OH-5), 8.60 (1H, s, H-3”), 7.37 (2H, s, H-2’ and H-6’), 7.16 (1H, s, H-3), 6.99 (1H, d, J = 2.1 Hz, H-8), 6.48 (1H, d, J = 2.1 Hz, H-6), 6.38 (1H, d, J = 9.0 Hz, H-1’”), 5.67 (1H, t, J = 9.5 Hz, H-2’”), 5.55 (1H, t, J = 9.5 Hz, H-3’”), 5.32 (2H, s, H-1”), 5.17 (1H, t, J = 9.8 Hz, H-4’”), 4.39-4.35 (1H, m, H-5’”), 4.13-4.10 (2H, m, H-6’”), 3.90 (6H, s, OCH_3, H-3’ and H-5’), 3.74 (3H, s, OCH_3, H-4’), 2.02 (3H, s, $COCH_3$), 2.00 (3H, s, $COCH_3$), 1.96 (3H, s, $COCH_3$), 1.78 (3H, s, $COCH_3$). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.2 (C4), 170.1 ($\underline{C}OCH_3$), 169.6 ($\underline{C}OCH_3$), 169.4 ($\underline{C}OCH_3$), 168.5 ($\underline{C}OCH_3$), 163.8 (C7), 163.2 (C2), 161.2 (C5), 157.2 (C8a), 153.3 (C3’ and C5’), 142.7 (C2”), 140.9 (C4’), 123.9 (C3”), 125.8 (C1’), 105.1 (C3 and C4a), 104.2 (C2’ and C6’), 98.8 (C6), 93.8 (C8), 83.9 (C1’”), 73.3 (C5’”), 72.1 (C3’”), 70.1 (C2’”), 67.5 (C4’”), 61.8 (C1” and C6’”), 60.3 (OCH_3), 56.3 (OCH_3), 20.6 ($CO\underline{C}H_3$), 20.5 ($CO\underline{C}H_3$), 20.3 ($CO\underline{C}H_3$), 19.9 ($CO\underline{C}H_3$).

4.2.6. Synthesis of flavone *O*-glycosides LuMeTGluOH and TriCeTGluOH

LuMeTGluAc/TriCeTGluAc (0.5 mmol) were treated with 0.5 M sodium methoxide (Sigma 403067, 1mL, 0.5 mmol) in 30 mL of methanol (dried over activated 4 Å molecular sieves) and the reaction mixture was stirred at room temperature for 2 h. The acidified resin was then added to the reaction mixture and was stirred for 2 h at room temperature. The reaction was filtered by cotton and, then, the solvent was evaporated and the pure product was obtained.

7-((*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)luteolin 3’,4’-dimethyl ether (LuMeTGluOH). Yield: 85%; mp 173 °C; IR (kBr) υmax: 3600-3200, 2919, 1664, 1518, 1500, 1457, 1326, 1259 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 300.13 MHz), δ: 12.91 (1H, s, OH-5), 8.49 (1H, s, H-3”), 7.69 (1H, dd, J = 8.5 and 2.1 Hz, H-6’), 7.57 (1H, d, J = 2.0 Hz, H-2’), 7.12 (1H, d, J = 8.5 Hz, H-5’), 7.03 (1H, s, H-3), 6.96 (1H, d, J = 2.1 Hz, H-8), 6.47 (1H, d, J = 2.1 Hz, H-6), 5.55 (1H, d, J = 9.3 Hz, H-1’”), 5.41 (1H, d, J = 5.7 Hz, OH-3’”), 5.28 (3H, s, H-1” and OH-6’”), 5.15 (1H, d, J = 5.7 Hz, OH-2’”), 4.63 (1H, d, J = 5.7 Hz, OH-4’”), 3.86 (3H, s, OCH$_3$, H-4’), 3.83 (3H, s, OCH$_3$, H-3’), 3.80-3.76 (1H, m, H-2’”), 3.52-3.48 (1H, m, H-4’”), 3.47-3.44 (1H, m, H-5’”), 3.46-3.42 (2H, m, H-6’”), 3.44-3.40 (1H, m, H-3’”). ^{13}C NMR (DMSO-d$_6$, 75.47 MHz) δ: 182.1 (C4), 163.9 (C7), 163.7 (C2), 161.2 (C5), 157.3 (C8a), 152.3 (C4’), 149.0 (C3’), 141.8 (C2”), 124.3 (C3”), 122.8 (C1’), 120.2 (C6’), 111.7 (C5’), 109.5 (C2’), 105.0 (C3), 104.1 (C4a), 98.6 (C6), 93.5 (C8), 87.5 (C1’”), 80.0 (C5’”), 77.0 (C3’”), 72.0 (C2’”), 69.6 (C4’”), 61.8 (C1”), 60.8 (C6’”), 55.9 (OCH$_3$), 55.8 (OCH$_3$).

7-((*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)tricetin trimethyl ether (TriCeTGluOH). Yield: 77%; mp 169 °C; IR (kBr) υmax: 3600-3200, 2919, 2850, 1662, 1593, 1504, 1463, 1352, 1253 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 300.13 MHz), δ: 12.75 (1H, s, OH-5), 8.40 (1H, s, H-3”), 7.27 (2H, s, H-2’ and H-6’), 7.05 (1H, s, H-3), 6.95 (1H, d, J = 1.8 Hz, H-8), 6.41 (1H, d, J = 1.8 Hz, H-6), 5.47 (1H, d, J = 9.0 Hz, H-1’”), 5.34 (1H, d, J = 5.5 Hz, OH-3’”), 5.20 (3H, s, H-1” and OH-6’”), 5.09 (1H, d, J = 5.7 Hz, OH-2’”), 4.57 (1H, d, J = 5.7 Hz, OH-4’”), 3.81 (6H, s, OCH$_3$, H-3’ and H-5’), 3.78-3.74 (1H, m, H-2’”), 3.65 (3H, s, OCH$_3$, H-4’), 3.50-3.45 (1H, m, H-4’”), 3.48-3.42 (1H, m, H-5’”), 3.43-3.39 (1H, m, H-3’”), 3.38-3.32 (2H, m, H-6’”). ^{13}C NMR (DMSO-d$_6$, 75.47 MHz) δ: 182.2 (C4), 164.1 (C7), 163.5 (C2), 161.2 (C5), 157.4 (C8a), 153.4 (C3’ and C5’), 141.9 (C2”), 141.0 (C4’), 125.9 (C1’), 124.4 (C3”), 105.4 (C3), 105.2 (C4a), 104.3 (C2’ and C6’), 98.8 (C6), 93.8 (C8), 87.6 (C1’”), 80.1 (C5’”), 77.1 (C3’”), 72.1 (C2’”), 69.9 (C4’”), 61.9 (C1”), 60.8 (C6’”), 60.4 (OCH$_3$), 56.4 (OCH$_3$).

4.2.7. Synthesis of flavone *O*-sulfate glycosides LuMeTGluS and TriCeTGluS

To a solution of **LuMeTGluOH/TriCeTGluOH** (150 mg, 0.27/0.26 mmol) in DMF (10 mL), triethylamine-sulfur trioxide adduct (Sigma 84739, 700 mg, 4 equi/OH) was added. The reaction, in open vessel, was kept stirring and heated for 30 minutes at 100 °C under MW irradiation of 200 W. After cooling, the mixture was poured into acetone (200 mL) under basic conditions (a few millilitres of triethylamine) and left at 4 °C overnight. The crude oil formed was washed with acetone and then dissolved in aqueous solution of 30% sodium acetate (1 mL). The suspension was added dropwise in ethanol to precipitate the sodium salt of the sulfated derivative. The solid obtained was further purified from other salts (monitored by IR) using a Spectra/Por 6 regenerated cellulose MWCO 1000.

7-((2,3,4,6-tetrasulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)luteolin 3’,4’-dimethyl ether (LuMeTGluS). Yield: 80%; mp 194 °C; IR (kBr) υmax: 3600-3300, 2919, 1660, 1517, 1503, 1455, 1260, 1017, 808 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 500.16 MHz), δ: 12.81 (1H, s, OH-5), 8.45 (1H, s, H-3”), 7.62 (1H, dd, J = 8.0 and 2.0 Hz, H-6’), 7.49 (1H, d, J = 2.0 Hz, H-2’), 7.04 (1H, d, J = 8.0 Hz, H-5’), 6.92 (1H, s, H-3), 6.90 (1H, d, J = 2.0 Hz, H-8), 6.40 (1H, d, J = 2.0 Hz, H-6), 5.74 (1H, d, J = 9.0 Hz, H-1’”), 5.25 (2H, s, H-1”), 5.00 (1H, t, J = 3.5 Hz, H-2’”), 4.60 (1H, t, J = 3.3 Hz, H-3’”), 4.40 (1H, t, J = 2.5 Hz, H-4’”), 4.27-4.23 (1H, m, H-5’”), 3.77 (3H, s, OCH$_3$, H-4’), 3.74 (3H, s, OCH$_3$, H-3’), 3.69-3.65 (2H, m, H-6’”). ^{13}C NMR (DMSO-d$_6$, 125.77 MHz) δ: 182.2 (C4), 163.8 (C7), 161.2 (C2), 157.4 (C5), 152.3 (C8a), 149.1 (C4’), 142.0 (C3’), 141.4 (C2”), 124.3 (C3”), 122.9 (C1’), 120.3 (C6’), 111.8 (C5’), 109.5 (C2’), 105.1 (C3), 104.1 (C4a), 98.8 (C6), 93.6 (C8), 86.9 (C1’”), 82.4 (C5’”), 77.1 (C3’”), 70.8 (C2’”), 68.7 (C4’”), 65.6 (C6’”), 61.9 (C1”), 56.0 (OCH$_3$), 55.8 (OCH$_3$).

7-((2,3,4,6-tetrasulfate-*β*-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl)methoxy)tricetin trimethyl ether (TriCeTGluS).

Yield: 77%; mp 172 °C; IR (kBr) υmax: 3600-3300, 2919, 1617, 1501, 1465, 1259, 1034, 802 cm^{-1}; ^{1}H NMR (DMSO-d$_6$, 500.16 MHz), δ: 12.84 (1H, s, OH-5), 8.54 (1H, s, H-3”), 7.40 (2H, s, H-2’ and H-6’), 7.14 (1H, s, H-3), 7.10 (1H, d, J = 2.1 Hz, H-8), 6.54 (1H, d, J = 2.1 Hz, H-6), 6.23 (1H, d, J = 9.3 Hz, H-1’”), 5.25 (2H, s, H-1”), 4.99 (1H, t, J = 3.6 Hz, H-2’”), 4.76 (1H, t, J = 3.5 Hz, H-3’”), 4.25 (1H, t, J = 3.0 Hz, H-4’”), 3.96-3.91 (1H, m, H-5’”), 3.82 (6H, s, OCH$_3$, H-3’ and H-5’), 3.76 (3H, s, OCH$_3$, H-4’), 3.65-3.60 (2H, m, H-6’”).

^{13}C NMR (DMSO-d$_6$, 125.77 MHz) δ: 182.3 (C4), 164.2 (C7), 163.5 (C2), 161.2 (C5), 157.5 (C8a), 153.4 (C3’ and C5’), 141.9 (C2’’), 140.9 (C4’), 126.0 (C1’), 124.5 (C3’’), 105.2 (C3 and C4a), 104.3 (C2’ and C6’), 98.9 (C6), 93.8 (C8), 87.5 (C1’’’), 83.9 (C5’’’), 78.7 (C3’’’), 71.7 (C2’’’), 69.0 (C4’’’), 66.6 (C6’’’), 62.0 (C1’’), 60.4 (OCH$_3$), 56.5 (OCH$_3$).

4.3. Antifouling Activity*

4.3.1. *Mytilus galloprovincialis* plantigrades collection and processing

M. galloprovincialis juvenile (0.5 cm shell length approximately) aggregates were collected from mussel beds from the intertidal rocky shore, during low neap tides at Memória beach, Matosinhos, Portugal, and immediately transported to the laboratory. *M. galloprovincialis* plantigrade post-larvae (0.5–2 mm) were screened among the small mussel aggregates in a binocular magnifier (Olympus SZX2-ILLT), gently washed with filtered seawater to remove organic debris and sand particles and isolated in a petri dish with filtered seawater immediately before the bioassays.

4.3.2. Antifouling bioassays

For the screening bioassay, competent *M. galloprovincialis* plantigrades (showing exploring behavior, ie, moving their foot searching for the appropriate substrate to settle) were selected and exposed to the series of compounds at 50 µM concentration in 24-well microplates for 15 h, at 18±1 °C, in the darkness. Bioassays time frame was selected based on preliminary bioassays to ensure that the response measured is reflecting the compounds effect. Test solutions were prepared in filtered seawater (previously treated by UV light, and carbon filters and mechanically filtered with 0.45 µM filter before use) and obtained by dilution of the compounds stock solutions (50 mM) in ultra-pure water. Four well replicates were used per condition with five plantigrades per well and 2.5 mL of test solution. A negative control, with ultra-pure water was included in all bioassays, as well as a positive control with 5 µM CuSO4 (a potent antifouling agent). At the end of the exposure period, the anti-settlement activity was determined by the presence/absence of byssal threads produced by each individual efficiently attached for all the conditions tested.

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*Work supervised by Joana R. Almeida, PostDoc Researcher in CIIMAR.

4.4 The environmental fate predictions

KOWWIN™ v1.68, WSKOWWINTM v1.42, and BIOWIN™ v4.10 developed by the U.S. EPA (http://www.epa.gov/oppt/exposure/pubs/episuite.htm) were used to predict bioaccumulation, water solubility, and biodegradability, respectively.

KOWWIN™ was used to estimate the log octanol-water partition coefficient log K_{ow}. Compounds were considered potentially bioaccumulative if the log K_{ow} was 3 or more.[72]

WSKOWWIN™ estimates the water solubility of an organic compound using the log K_{ow} of compounds.

BIOWIN™ utilizes functional group contribution approaches in the estimation of aerobic biodegradation of organic compounds in the presence of microorganisms in the environment. Within BIOWIN™ seven separate models were used: BIOWIN™ 1 - linear, BIOWIN™ 2 - non-linear, BIOWIN™ 3 - ultimate, BIOWIN™ 4 - primary, BIOWIN™ 5 - linear Ministry of International Trade and Industry (MITI), BIOWIN™ 6 - non-linear MITI, and BIOWIN™ 7 – anaerobic. For a compound to be considered as biodegradable it must obay to one of the following criteria: Criteria A- BIOWIN™ 2 or 6 > 0.5, and BIOWIN™ 3 > 2.2; Criteria B- BIOWIN™ 3 "weeks" or faster, and BIOWIN™ 5 > 0.5. The BIOWIN™ program automatically generates a yes/no qualitative prediction of ready biodegradability.[72]

CHAPTER V

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APPENDIX I

Marine natural flavonoids: chemistry and biological activities

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Abstract

As more than 70% of the world's surface is covered by oceans, marine organisms offer a rich and unlimited resource of structurally diverse bioactive compounds. These organisms have developed unique properties and bioactive compounds that are, in majority of them, unparalleled by their terrestrial counterparts due to the different surrounding ecological systems. Marine flavonoids have been extensively studied in the last decades due to a growing interest concerning their promising biological/pharmacological activities. The most common classes of marine flavonoids are flavones and flavonols, which are mostly isolated from marine plants. Although most of flavonoids are hydroxylated and methoxylated, some marine flavonoids possess an unusual substitution pattern, not commonly found in terrestrial organisms, namely the presence of sulfate, chlorine, and amino groups. Marine flavonoids have demonstrated interesting biological activities, such as antioxidant, antimicrobial (antibacterial and antiviral), antitumor, anticoagulant, antidiabetic, antifouling, and antifeedant.

This review presents, for the first time in a systematic way, the structure, natural occurrence, and biological activities of marine flavonoids.

Keywords: marine flavonoids; biological activities; marine sources.

Abbreviations:

ABTS 2,2'-Azino-bis[3-ethylbenzothiazoline-6-sulphonate] radical

CC_{50} Half cytotoxic concentration

DPPH α,α-Diphenyl-β-picrylhydrazyl radical

HIV Human immunodeficiency virus

MIC Minimum inhibitory concentration

MMPs Matrix metalloproteinases

MTT Methyl Thiazol Tetrazolium

PMA 12-Myristate 13-acetate

ROS Reactive oxygen species

Introduction

Marine natural products have attracted the attention of biologists and chemists all over the world. Marine flora and fauna represent a virtually untapped resource for identifying and extracting bioactive natural products with potential to act as nutritional supplements, pharmaceuticals, cosmetics, agrochemicals, molecular probes, enzymes, and fine chemicals (Suleria et al., 2016).

Flavonoids are low molecular weight secondary metabolites not only commonly found in terrestrial plants (Kumar et al., 2013; Romano et al., 2013), but also found in marine sources. Since the discovery of the first marine flavonoids in 1966 (Utkin, 1966), almost 100 flavonoids have been isolated. Recent studies have revealed that marine flavonoids exhibit a wide spectrum of beneficial biological activities (Bao et al., 2013; Behery et al., 2012; Feng et al., 2006; Hawas, 2014; Hou et al., 2016; Jensen et al., 1998; Jiang et al., 1997; Ma et al., 2017; Mohammed et al., 2014; Park et al., 2015; Qi et al., 2008; Rodrigues et al., 2015; Rowley et al., 2002), and therefore these natural products have attracted much attention.

Over the past 50 years, the reports about marine flavonoids isolation have been increasing, especially flavones (68 compounds) and flavonols (23 compounds). It is noteworthy that the newly identified marine flavones have been mainly reported in the last decade (**Table 1**). Otherwise, flavonoids of other classes are quite rare, being isolated only two flavan-3-ols, two dihydrochalcones, one flavanone, and one isoflavone.

Insert Table 1

In spite of the variety and structural diversity of marine flavonoids, they have been isolated from a limited number of families especially the Hydrocharitaceae, Zosteraceae, Rhodomelaceae, Juncaceae, and Tamaricaceae (**Table 2**).

Insert Table 2

Most of flavonoids have been isolated from seagrass (35%), angiosperms (25%), and halophytes (18%). Nevertheless, the isolation of these natural products have also been reported from other marine sources, such as mangroves, algaes, molluscs, fungus, corals, and bacteria (**Figure 1**).

Insert Figure 1

Flavones

The first report about marine flavones concerns the isolation of four flavone *C*-glycosides (**Figure 2**) from the green alga *Nitella Hookeri* (Characeae). According to Markham and Porter (Markham et al., 1969) these flavones appear to be vicenin and lucenin types. Nevertheless, insufficient amount of sample was obtained to perform their complete characterization by NMR studies. Thereafter, more 64 flavones have been described, making flavones as the most representative class of marine flavonoids (**Table 1**).

Insert Figure 2

The majority of marine flavones possess hydroxyl and methoxy groups, including 33 glycosides (**15-17, 28-39, 41-47, 51, 54, 55, 57-64**), and 31 aglycones (**1-14, 18-27, 40, 48-50, 52, 53, 56**), making these substituents the most commonly found in this family of marine compounds (**Table 3**).

Insert Table 3

Table 4 summarizes the structure, natural occurrence, and biological activities of marine flavones organized by chronological order of the year in which they were first isolated. All flavones listed in **Table 4** are polyhydroxylated, being particularly common the presence of three and four hydroxyl groups at positions 5,3',4' (**2, 15, 19, 26, 51, 61**), 5,7,4' (**8, 11, 14, 18, 27, 38, 57, 59**) and 5,7,3',4' (**22, 58, 60**). Flavones with five (**6, 36, 37**) and six hydroxyl groups (**23**) have also been isolated from marine sources. Interestingly, the presence of flavones with hydroxyl and methoxy groups at less common positions, such as C-6 (**8, 11-14, 23, 25, 26, 29-37, 40, 52**) and C-8 (**52-55**) is also observed.

Considering methylated flavones, the majority are *O*-methylated, whereas *C*-methylation is quite rare. Only two *C*-methylated marine flavones are known (**52, 56**), both on B ring. Almost 70% of the flavones are mono-*O*-methylated (**3, 4,** 7, **8, 16, 19, 21, 24-27, 40, 43, 45-47, 63, 64**), being this group more frequently found on positions 7 (**19, 24-26, 46, 47**) and 3' (**4, 16, 27, 43, 45, 63**). In addition, 20% consist of di-*O*-methylated (**9-13, 20**) and the remaining 10% are tri-*O*-methylated flavones (**21, 48, 49**).

Among the 33 flavone glycosides, the majority are *O*-glycosides and only four are *C*-glycosides (**57-60**). From the 29 flavonoids *O*-glycosides, about two-third are 7-*O*-glucosides (**15-17**, **28-37**, **41-43**, **51**, **61-64**). The remaining consists of five 4'-*O*-glucosides (**39**, **44-47**), two 7-*O*-xylosides (**54**, **55**), and one 3'-*O*-glucoside (**38**).

As it happens with other marine secondary metabolites, some flavones also revealed a unique substitution pattern, namely the presence of a 6,8-dihydroxylated A ring (flavone **52**), a pyrrolidinyl ring (**14**), and sulfate groups linked either to flavone aglycones (**1-5**) or to the sugar moiety of flavone glycosides (**15-17**, **55**). In addition, a flavone with a more complex substitution pattern such as hydnocarpin (**50**), structurally related to the flavonolignan isosilybin, has also been found in the marine plant *Juncus acutus* (Behery et al., 2013).

Among all marine flavones, 24 have been exclusively extracted from the marine environment, particularly, from plants of Hydrocharitaceae family (**3-5**, **9**, **10**, **14**, **29-33**, **35-37**, **57-60**) seagrass (**15-17**, **55**), fungus (**56**), and a coral (**52**).

Insert Table 4

Flavonols

Flavonols represent the second most abundant class of marine flavonoids (**Table 1**). As for flavones, the majority of flavonols are polyhydroxylated, including 14 flavonolosides (**66, 67, 68, 73-75, 77-82, 84, 85**) and 9 aglycones (**65, 69-72, 76, 83, 86, 87**). More than two-thirds of these compounds are trihydroxylated at positions 5,7,4' (**66, 71, 73, 78, 79, 81, 83**) and tetrahydroxylated at positions 5,7,3',4' (**67, 74-77, 80, 82, 84, 85**). Most flavonols possess hydroxyl and methoxy groups being trisubstituted at 5,7,4' (**69-71, 73, 77, 81, 83**) and tetrasubstituted at 5,7,3',4' (**65, 66, 74-76, 78, 84, 85**) (**Tables 5 and 6**).

Insert Table 5

In addition to these substituents, the presence of substitution patterns not commonly found in terrestrial world such as methyl and sulfate (**65, 70, 72**) groups, as well as chlorine atom at C-3' (compound **86**) has been reported.

Flavonols glycosides have been frequently isolated, being almost all 3-*O*-glycosides (**66, 67, 73-75, 77-82, 84, 85**), with glucose as the predominant sugar (**68, 75, 77-80**). Flavonols with other sugars at the same position, such as rutinose (**66, 67**), fucose (**73, 74**), galactose (**81, 82**), arabinose (**84**), and rhamnose (**85**) have also been isolated. Only one hydroxylated flavonoloside with a 3'-*O*-glycoside (**68**) has been reported.

Regarding sulfated flavonols, three monosulfated compounds with sulfate groups at C-3 (**65, 70**) and C-3' (**71**) have been isolated from *Tamarix laxa* (Utkin, 1966), *Tamarix gallica* (Tomás-Barberán et al., 1990), and *Hypericum elodes* (Seabra et al., 1991), respectively, and two trisulfated flavonols with sulfate groups at C-3,5,4' (**68**) and C-3,3',4' (**72**) from *Tamarix aphylla* (Saleh et al., 1975) and *Tamarix amplexicaulis* (Barakat, 1998), respectively.

Among the flavonols isolated from marine sources, rhamnetin-3,5,4'-*O*-trisulfate-3'-*O*-*β*-D-glucuronopyranosyl (**68**) have been exclusively reported in the marine environment.

Insert Table 6

Other flavonoids

Flavonoids belonging to other classes like flavanones, flavan-3-ols, isoflavones, and dihydrochalcones have also been isolated from marine environment (**Tables 1 and 7**). Among these flavonoids, actinoflavoside (**88**) and thalassodendrone (**93**) are flavonoids with an unique substitution pattern not found in terrestrial world. Actinoflavoside (**88**) is a flavanone isolated from *Streptomyces sp*. (Jiang et al., 1997) showing an aminodeoxy sugar moiety at C-7. Thalassodendrone (**93**) is a dihydrochalcone isolated from *Thalassodendrin ciliatum* (Mohammed et al., 2014) that have a 6'-*O*-rhamnosyl-(1'''→ 6'')-glucopyranosyl as substituent group.

Insert Table 7

From around 93 flavonoids isolated from marine sources, 27 have only been reported in the marine environment (**3-5, 9, 10, 14-17, 29-33, 35-37, 52, 55-60**, **68, 88, 93**), reinforcing the importance of this habitat to the discovery of flavonoids with an unique structure.

Biological activities of marine natural flavonoids

Some marine natural flavonoids have been attracting the scientific community because of their unique substitution pattern and promising biological activities. In fact, several marine flavonoids have been reported has having not only pharmacological activities (antioxidant, antimicrobial, antitumor, anticoagulant, and antidiabetic activities), but also antifouling and antifeedant activities, demonstrating their potential as lead compounds for pharmaceutical and chemical industries. Among these flavonoids some have also been isolated from terrestrial sources and are commonly found in diet, such as apigenin, isorhamnetin, kaempferol, luteolin, and quercetin (Beecher, 2003). The biological activities described for these terrestrial flavonoids have been extensively reviewed by several researchers (Birt et al., 2001; D'Andrea, 2015; Ghasemzadeh et al., 2011; Seelinger et al., 2008). In this section the biological activities described in research papers focusing flavonoids isolated from marine organisms (**Tables 4**, **6**, and 7) will be discussed.

It is noteworthy that from the 27 flavonoids exclusively isolated from marine environment, only eight were studied for their biological activities. Developing drugs from marine sources demands suitable amounts of the compound under study, which often cannot be met by re-isolation from the respective marine source. In this direction, the search for alternatives for resupply unique structure as flavonoids **3-5**, **9**, **10**, **14**, **29-33**, **35-37**, **57-60**, **68**, namely biotechnology approaches and/or total organic synthesis, worth to be explore in the future.

Antioxidant activity

Terrestrial flavonoids have been reported to act as scavengers of several oxidising species, namely reactive oxygen species (ROS), such as superoxide anion, hydroxyl, and peroxyl radicals, and may also act as quenchers of singlet oxygen (Harborne et al., 2000). However, the reports about the antioxidant activity of marine flavonoids are limited to the study of their scavenging effect against *α*,*α*-diphenyl-*β*-picrylhydrazyl (DPPH) and 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonate] (ABTS) radicals, and by electron spin resonance spectroscopy.

Based on the experiments with DPPH radical-scavenging assay, Feng *et al.* demonstrated that apigenin (**18**) and 5,3',4'-trihydroxy-7-methoxyflavone (**19**), isolated from the aerial parts of the mangrove plant *Avicennia marina,* showed moderate DPPH scavenging activity (IC_{50} = 52.0 and 37.0 μg.ml^{-1}, respectively) when compared to the positive control (IC_{50} = 18.0 μg.ml^{-1}), butylated hydroxytoluene (BHT), a well-known synthetic antioxidant (Feng et al., 2006).

Behery *et al.* evaluated the antioxidant activity of apigenin (**18**), luteolin (**22**), chrysoeriol (**27**), hydnocarpin (**50**), and luteolin-7-*O*-*β*-D-glucoside (**51**), isolated from the halophyte *Juncus acutus L.*, using ABTS radical cation decolourisation assay (Behery et al., 2012). Results showed that the potency of antioxidant activity of compounds was found to be dependent of the number of free phenolic groups in their molecules. In fact, while luteolin (**22**), with four phenolic groups, showed the highest scavenging activity (90.7 % at 1 mg.ml^{-1}), apigenin (**18**), chrysoeriol (**27**), hydnocarpin (**50**), and luteolin-7-*O*-*β*-D-glucoside (**51**), with three phenolic groups, showed only moderate scavenging activity (68-75 % at 1 mg.ml^{-1}) (Behery et al., 2012).

To evaluate the scavenging effect of isorhamnetin 3-*O*-*β*-D-glucoside (**78**) and respective demethyl derivative, quercetin 3-*O*-*β*-D-glucoside (77), isolated from *Salicornia herbacea,* hydroxyl radicals were generated in a cell free system and analyzed by electron spin resonance spectroscopy. Both compounds revealed an excellent radical scavenging ability (80 and 94% inhibitory activities, respectively, at concentrations of 10 μM) when compared with control group (100 % of inhibitory activity) (Kong et al., 2008).

The overall results presented for marine flavonoids with antioxidant activity are in agreement with the results described for other structure related flavonoids. In fact, it has been proposed that the number and the position of hydroxyl groups in the flavone scaffold influence their scavenging activity, being the presence of catechol groups favorable to this activity (Amić et al., 2007).

Antimicrobial activity

Marine flavonoids were shown to be effective against bacteria, and virus (Hawas, 2014; Jiang et al., 1997; Mohammed et al., 2014; Qi et al., 2008; Rowley et al., 2002).

Qi *et al.* investigated the antibacterial activity of luteolin (**22**) and luteolin-4'-glucuronide (**39**), isolated from ethanol extracts of the South China Sea seagrass *Enhalus acoroides*, towards several marine pathogenic bacteria (*Pseudoalteromonas piscida*, *Vibrio alginolyticus,* and *Vibrio furnissii*). Luteolin (**22**) had slight inhibition (MIC = 50 μg.ml-^{1}) towards *P. piscida* and *V. alginolyticus*, while luteolin 4'-glucuronide (**39**) inhibited the growth of the bacteria *V. alginolyticus* and *V. furnissii* at high concentrations (200 μg.ml-

^{1}) (Qi et al., 2008). Overall results suggest that luteolin (**22**) can be a lead compound to the discovery of new antibacterial agents useful for protection against marine pathogenic bacteria.

The antibacterial activity of isoscutellarein (**53**), isoscutellarein-7-*O*-xylopyranoside (**54**), and isoscutellarein 7-*O*-*β*-xylopyranoside-2”-*O*-sulfate (**55**), isolated from seagrass *Thalassia hemprichii,* against *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 5141), *Escherichia coli* (ATCC 10536), and *Pseudomonas aeruginosa* (NTCC 6750) were investigated by Hawas *et al.* (2014). The compounds were subjected to agar diffusion assays for antimicrobial activity and the minimum inhibitory concentration (MIC) obtained was in a range between 0.1–10 μg.ml^{-1} against Gram-positive and Gram-negative bacteria. Isoscutellarein 7-*O*-*β*-xylopyranoside-2”-*O*-sulfate (**55**) exhibited potent activity against *Bacillus subtilis* (MIC = 2.5 μg.ml^{-1}) and *Pseudomonas aeruginosa* (MIC = 1.5 μg.ml^{-1}), while isoscutellarein (**53**) and isoscutellarein-7-*O*-xylopyranoside (**54**) showed weak activity (MIC = 3.2-9.4 μg.ml^{-1}).

Using the agar diffusion method, Jiang *et al.* studied the antibacterial activity of actinoflavoside (**88**), isolated from *Streptomyces* sp. of saline environments, at 3 mg.l^{-1}, against Gram-positive bacteria, including *Staphylococcus pneumonia*, *Staphylococcus pyrogenes*, *Staphylococcus aureus*, and *Micrococcus luteus* (Jiang et al., 1997). This flavanone showed weak antibacterial activity against all tested Gram-positive bacteria, revealing MIC of 64 μg.ml^{-1} for all strains (Jiang et al., 1997).

Investigation of the antiviral activity of marine flavonoids has been focused on human immunodeficiency virus (HIV) and on virus influenza A. Thalassiolins A–C (**15-17**), isolated from the Caribbean seagrass *Thalassia testudinum*, were studied as inhibitors of HIV integrase. All tested compounds displayed *in vitro* inhibition of the integrase catalyzed strand transfer reaction, being thalassiolin A (**15**) the most potent compound (Thalassiolin A: IC_{50} = 0.4 μg.ml^{-1}, Thalassiolin B: IC_{50} = 43 μg.ml^{-1}, Thalassiolin C: IC_{50} = 28 μg.ml^{-1}) (Rowley et al., 2002).

More recently, the antiviral influenza A activity of asebotin (**92**) and thalassodendrone (**93**), isolated from seagrass *Thalassodendrin ciliatum*, was evaluated using the Methyl Thiazol Tetrazolium (MTT) method (Mohammed et al., 2014). Both compounds revealed antiviral activity, being compound **93** (IC_{50} =1.96 μg.ml^{-1}) the most potent (IC_{50} = 2.00 μg.ml^{-1}). Moreover, the cytotoxic effect based on the viability of the mock-infected cells of each compound was monitored by the MTT method. Asebotin (**92**) presented a higher half cytotoxic concentration (CC_{50} =3.36 μg.ml^{-1}) than thalassodendrone (**93**) (CC_{50} = 3.14 μg.ml^{-1}) (Mohammed et al., 2014).

Antitumor activity

The antitumor activity of marine flavonoids also reported from terrestrial world have been exhaustively studied. In fact, several flavonoids such as apigenin (**18**), luteolin (**22**), 3',5'-dihydroxyscutellarein (**23**), chrysoeriol (**27**), quercetin-3-*O*-rutinoside (**67**), quercetin (**76**), kaempferol (**79**), and (-)-catechin (**85**) have shown to inhibit the growth of human tumor cell lines, being this effect associated with the interference of several molecular targets (Batra et al., 2013; Chahar et al., 2011; Ren et al., 2003). Nevertheless, the antitumor potential of flavonoids only reported in the marine environment is still scarce.

Hou *et al*, assayed the *in vitro* cytotoxic activity of penimethavone (**56**) against cervical (HeLa), human laryngeal epithelial (Hep-2), rhabdomyosarcoma (RD), and non-small cell lung cancer (A549) cell lines, using adriamycin as a positive control. Penimethavone showed selective and moderate cytotoxicity against HeLa (IC_{50} = 8.41 μg.ml^{-1}) and RD (IC_{50} = 8.18 μg.ml^{-1}) cells (Hou et al., 2016).

Matrix metalloproteinases (MMPs), particularly, MMP-9 and MMP-2, are principal enzymes in extracellular matrix degradation and play an important role in several pathologies, including cancer (Kong et al., 2008). Kong *et al.* isolated quercetin-3-*O*-*β*-D-glucoside (77) and isorhamnetin-3-*O*-*β*-D-glucoside (**78**) from *Salicornia herbacea* and studied their inhibitory effects on MMP-9 and MMP-2 in human fibrosarcoma cell line (HT1080) (Kong et al., 2008). Both flavonols reduced the expression levels and activities of

MMP-9 and MMP-2 (around 60%), at 10 µM, when compared with phorbol 12-myristate 13-acetate (PMA). In order to confirm that these flavonoid glycosides exert their inhibitory effects by interfering with AP-1 transcription factor binding, promoter activity of AP-1 was studied by transfection of the HT 1080 cells with AP-1 binding site containing luciferase constructs (Kong et al., 2008). The reporter activity was enhanced in the PMA stimulated group up to 2-fold, whereas the reporter activity was suppressed by treatment with these flavonoid glycosides. Therefore, the inhibitory effect of these flavonoid glycosides on MMP-9 and MMP-2 could be explained by the down-regulation of those genes *via* the transcription factor AP-1. However, comparative analysis did not exhibit any significant difference on AP-1 luciferase activity between these two flavonoid glycosides. So, these results suggested the potential of these flavonol glycosides (77 and **78**) as chemopreventive agents (Kong et al., 2008).

Anticoagulant activity

The enzyme thrombin is involved in the final coagulation cascade, being responsible for the formation of fibrin clots, the conversion of fibrinogen into fibrin, and the activation of other substrates (factors V, VIII, XI, and XIII). Thrombin can also mediate additional clotting events and enhance the efficiency of coagulation and peeling, being the direct inhibition of this enzyme an effective anticoagulant strategy (Correia-da-Silva et al., 2011). Rodrigues *et al.* demonstrated that the extract of white mangrove (*Laguncularia racemosa*) leaves significantly inhibited human thrombin activity and the coagulation of plasma in the presence of this enzyme (Rodrigues et al., 2015). A bioassay-guided chemical fractionation of this extract resulted in the identification of quercetin-3-*O*-arabinoside (**84**) and quercetin-3-*O*-rhamnoside (**85**) as the most potent inhibitors of human thrombin activity. HPLC analyses of the thrombin samples incubated with these compounds revealed their ability to interact with thrombin. In fact, under identical chromatographic conditions, native thrombin was eluted at 28.1 min, while thrombin treated with each flavone **84** and **85** was eluted at 25.2 and 23.8 min, respectively, suggesting that both of these flavonoids significantly decreased thrombin hydrophobicity. The effectiveness of theses flavonoids to promote thrombin inhibition was also confirmed using enzymatic assays (Rodrigues et al., 2015).

Antidiabetic activity

Several *in vitro* and *in vivo* studies have supported a beneficial effect of dietary flavonoids on glucose homeostasis, through several mechanisms, including the decrease of carbohydrate digestion as a result of α-glucosidase inhibition (Babu et al., 2013). Nevertheless, only two marine flavonoids with this activity have been reported. Ma *et al*, tested the *in vitro α*-glucosidase inhibitory activity of aspergivone A (**82**) and B (**83**), isolated from the fungus *Aspergillus candidus* cultured from the gorgonian coral *Anthogorgia ochracea* collected from the South China Sea, using deoxynojirimycin (IC_{50} = 42.40 μg.ml^{-1}), acarbose (IC_{50} = 457 μg.ml^{-1}), and miglitol (IC_{50} > 500 μg.ml^{-1}) as positive controls (Ma et al., 2017). Both flavones revealed a slight inhibitory activity, showing compound **83** an IC_{50} value of 244 μg.ml^{-1} and compound **82** showed only 30% of inhibition at 100 μg/ml (Ma et al., 2017).

Antifouling activity

The formation of biofouling involves the attachment of a range of micro- and macroorganisms in natural and artificial underwater surfaces, and causes serious technical and operational challenges for the maritime industries (Qian et al., 2015). Considering this, efforts have been on the development of nontoxic and environmentally friendly antifouling agents, which inhibit the biofilm formation and protect against marine biofouling organisms, such as pathogen bacteria, and other intruders. These agents should be capable to inhibit the settlement of selected biofouling species by acting in more specific signalling targets, somehow related with settlement processes, instead of inducing general toxicity (Qian et al., 2015).

In the past decades, several marine natural products have been reported as non-toxic antifouling compounds (Qian et al., 2015; 2009). From them, eight flavonoids with different substitution pattern were reported. Jensen *et al.* (1998) demonstrated that the crude extract of healthy *Thalassia testudinum* leaf tissues inhibited the growth of the co-occurring thraustochytrid *Schizochytrium aggregatum* and prevented the attachment of *S. aggregatum* motile zoospores to an extract-impregnated substrate (Jensen et al., 1998). A bioassay-guided chemical fractionation of this extract resulted in the isolation of thalassiolin A (luteolin-7-*O*-*β*-D-glucopyranosyl-2”-sulfate, **15**), a new sulfated flavone glycoside, which displayed antibiotic activity against *S. aggregatum* with an IC_{50} of 270 μg.ml^{-1}, and reduced the attachment of *S. aggregatum* motile zoospores to an extract-impregnated substrate. These results suggest the ecological role of thalassiolin A in chemical defense against fouling microorganisms, in addition to the antiviral activity as inhibitor of HIV integrase (Jensen et al., 1998).

More recently, two thalassiolin A structure related flavones, Luteolin (**22**) and luteolin-4’-glucuronide (**39**), isolated from the seagrass *Enhalus acoroides*, also revealed antibacterial activity against bacterial strains isolated from marine biofilms (*Loktanella hongkongensis, Rhodovulum* sp, and *Vibrio halioticoli*) (Qi et al., 2008). Luteolin (**22**) inhibited the growth of *L. hongkongensis* with a MIC of 50 μg.ml^{-1} and had slight inhibition towards *Rhodovulum* sp., while luteolin 4’-glucuronide (**39**) inhibited the growth of the bacteria *L. hongkongensis* and *V. halioticoli* at high concentrations (200 μg.ml^{-1}) (Qi et al., 2008). In addition to the antibacterial activity, compound **39** inhibited larval settlement of *Bugula neritina* effectively, with an half maximal effective concentration (EC_{50}) value of 0.52 μg.ml^{-1}, with low toxicity (Qi et al., 2008). Overall results suggest that luteolin (**22**) can be a lead compound to the discovery of new antifouling agents.

In 2013, Bao *et al.* studied the ethyl acetate extract of a culture broth of the marine-derived fungus *Penicillium* sp., isolated from South China Sea gorgonian coral *Dichotella gemmacea*, and concluded that this extract exhibited significant antifouling activity against *Balanus amphitrite* larvae settlement. Further investigation on the chemical constituents of the extract led to the isolation of a new flavone, 6,8,5',6'-tetrahydroxy-3'-methylflavone (**52**), with potential antifouling activity. Compound **52** strongly inhibited the *B. amphitrite* larvae settlement with EC_{50} value of 6.71 µg.ml^{-1}, showing no toxicity toward larvae at concentrations of 3.125–100 µg.ml^{-1}, which indicated that the LC_{50}/EC_{50} values was > 14 (Bao et al., 2013).

Kong *et al.* (2014) studied the antibacterial activity of flavonoids isolated from the leaves of the halophyte *Apocynum venetum* against marine biofilm-derived bacteria *Bacillus thuringiensis*, *Pseudoalteromonas elyakovii*, and *Pseudomonas aeruginosa*. 8-Methylretusin (**91**) showed weak antibacterial activity towards *B. thuringiensis* and *P. aeruginosa* (inhibition zone diameter of 9.4±0.6 and 10.5±0.9 mm, respectively), but no activity against *P. elyakovii*. Quercetin (**76**) and (-)-catechin (**89**) displayed weak antibacterial activity against all tested bacteria (inhibition zone diameter between of 9.2±0.5 and 10.9±0.7 mm), while kaempferol (**83**) exhibited moderate antibacterial activities towards all tested bacteria (*B. thuringiensis*: inhibition zone diameter of 17.4±0.9 mm, *P. elyakovii*: inhibition zone diameter of 9.5±0.6 mm, *P. aeruginosa:* 15.8±0.8 mm) (Kong et al., 2014).

Antifeedant activity

The use of antifeedants in pest-management programs has enormous potential because they satisfy the need to protect specific crops while avoiding damage to non-target organisms. The chemosensory mechanism governing feeding behaviour in insects is sensitive to a wide range of such constituents commonly known as feeding-deterrents. These substances reduce or prevent feeding by rendering plants unpalatable or toxic and thereby offer a novel approach for insect-pest and vector management. The use of natural products as insect control agents, which influence the chemosensory behaviour of insects, is a promising approach for insect-pest management (Jain et al., 1993). However, there are still few studies that refer to the use of flavonoids as antifeedants.

Flavonoids isolated from the South China seagrass *Enhalus acoroides* were evaluated for their antifeedant activity, by the conventional leaf disk method, against second-instar larvae of *Spodoptera litura*, which is a major vegetable pest in summer and fall in China (Qi et al., 2008). Additionally, the cytotoxicity of these secondary metabolites towards *S. litura* cells was also assessed to determine their insecticidal activity. Apigenin (**18**) showed significant antifeedant activity (80.6%), while luteolin (**22**) and luteolin-4'-glucuronide (**39**) exhibited only moderate activities (77.5 and 65.9%, respectively) when compared with the positive control azadirachtin (96.9%) at 500 $\mu g.ml^{-1}$ (Qi et al., 2008).

Conclusions and perspectives

The investigation of marine world is growing due to the fact that it is one of the largest biological resources, containing a vast array of organisms with unique biological systems and characteristics that are important sources of bioactive natural products. Among these natural products, some marine flavonoids with promising biological activity have been isolated.

This review provided an overview of the most relevant classes of marine flavonoids with flavones and flavonols being more representatives, mainly extracted from plants, like seagrass, angiosperm, halophytes, and mangroves, with a highly incidence of Hydrocharitaceae family. The most abundant flavones are hydroxylated flavone glycosides and hydroxylated *O*-methylated flavones, while the majority of flavonols are hydroxylated flavonolosides. Nevertheless, some flavonoids with distinctive substitution pattern have been isolated reinforcing the importance of this habitat to the discovery of flavonoids with a unique structure.

Marine natural flavonoids have been attracting attention of the scientific community not only by their promising biological activities, namely antioxidant, antitumor, antimicrobial (antibacterial and antiviral), anticoagulant, and antidiabetic, but also as antifouling and antifeedant agents. Although several studies about biological potential of these marine natural products have been reported, most of the flavonoids exclusively isolated from marine environment have not been completely explored concerning their biological potential. Therefore, it is expected that the future exploitation of these marine natural products may contribute to Medicinal Chemistry in the discovery of innovative drugs with new mechanisms of action.

Ackwnoledgments

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Figures captions

Figure 1 - Marine organisms as sources of flavonoids.

Figure 2 – Structure of lucenin and vicenin.

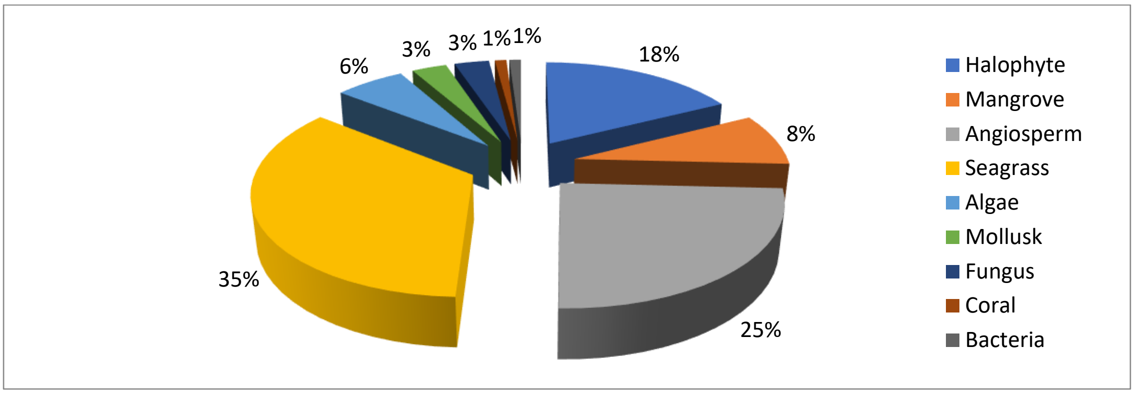

18%
8%
25%
35%
6%
3%
3%
1%
1%
Halophyte
Mangrove
Angiosperm
Seagrass
Algae
Mollusk
Fungus
Coral
Bacteria

Figure 1

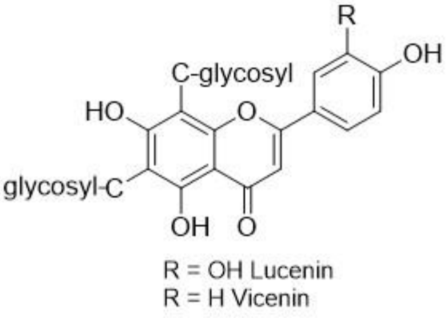


Figure 2

Table 1 – Numbers of flavonoids reported since 1966.

	Flavones	Flavonols	Flavanones	Flavan-3-ols	Isoflavones	Dihydrochalcones	Total
1966-76	9	4	-	-	-	-	13
1977-87	9	-	-	-	-	-	9
1988-98	1	3	1	-	-	-	5
1999-09	25	6	-	1	-	-	32
2010-17	24	6	-	1	1	2	34

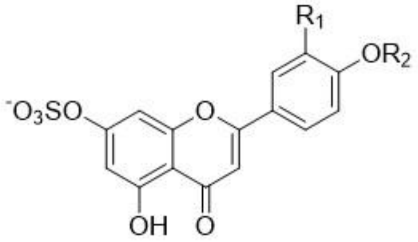
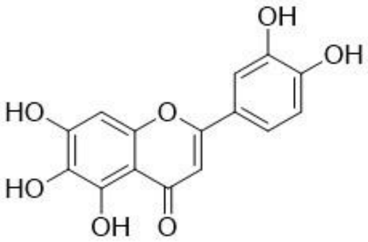
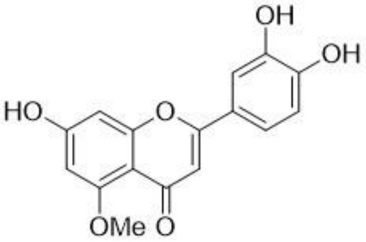
Table 2 – Distribution of marine flavonoids.

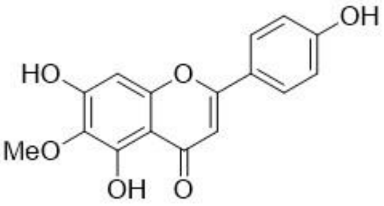
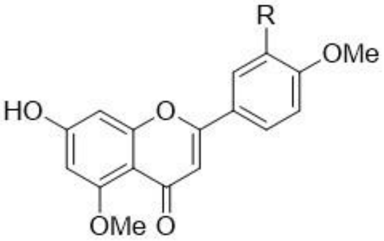
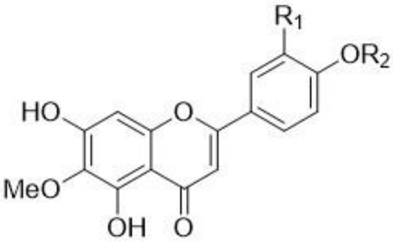
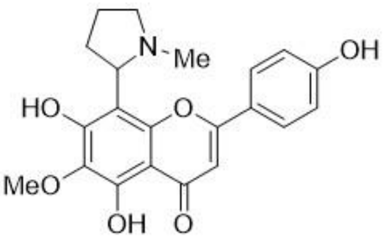
More representative families				
Hydrocharitaceae (33%)	Zosteraceae (17%)	Rhodomelaceae (7%)	Juncaceae (6%)	Tamaricaceae (6%)
Halophila johnsonii (15 compounds) *Halophila stipulacea* (6 compounds) *Thalassia hemprichii* (5 compounds) *Enhalus acoroides* (4 compounds) *Thalassia testudinum* (3 compounds)	*Zostera marina* (9 compounds) *Phyllospadix japonica* (8 compounds)	*Acanthophora spicifera* (5 compounds) *Osmundea pinnatifida* (1 compound)	*Juncus acutus* (5 compounds)	*Tamarix aphylla* (2 compounds) *Tamarix amplexicaulis* (1 compound) *Tamarixgallica* (1 compound) *Tamarix laxa* (1 compound)
Other families				
Amaranthaceae, Aplysiidae, Apocynaceae	Avicenniaceae, Bataceae, Characeae	Combretaceae, Cymodoceaceae, Caryophyllaceae,	Guttiferae, Juncaceae, Pellicieraceae	Rhodomelaceae, Streptomycetaceae, Tamaricaceae, and Trichocomaceae

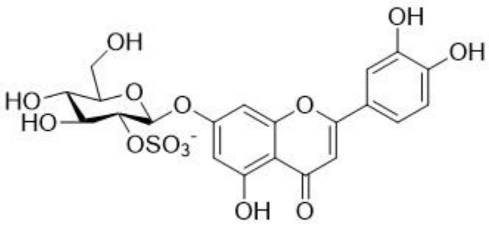
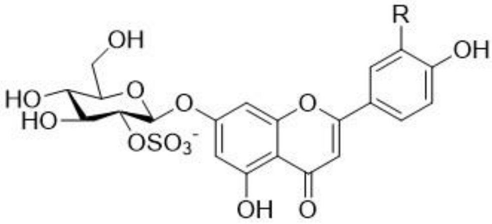
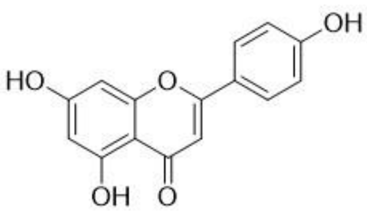
Table 3 – Frequency and substitution pattern of hydroxyl and methoxy groups among marine flavones.

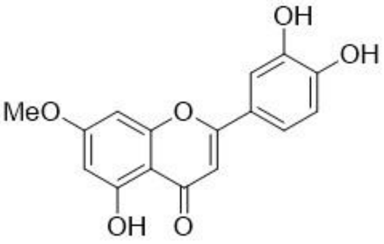
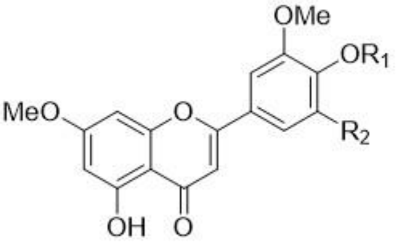
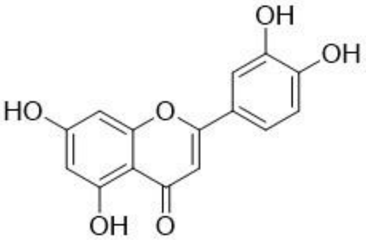
	7	**5,7**	**5,4’**	**5,6, 4’**	**5,7, 3’**	**5,7, 4’**	**5,8, 4’**	**5,3’, 4’**	**7,8, 4’**	**5,6, 7,4’**	**5,6, 3’,4’**	**5,7, 8,2’**	**5,7, 8,4’**	**5,7, 3’,4'**	**5,6,7, 3’,4’**	**5,6,3’,4’,5’**	**5,7,2’, 4’,6’**	**5,7,3’, 4’,5’**	**6,8,3’,5’,6’**	**5,6,7,3’,4’,5’**
Flavone aglycones	-	-	-	-	-	3	-	-	-	5	-	-	1	9	4	-	1	1	1	1
Flavone *C*-glycosides	-	-	-	-	-	2	-	-	-	-	-	-	-	2	-	-	-	-	-	-
Flavone *O*-glycosides	-	3	4	3	2	1	1	5	-	-	4	-	-	-	-	2	-	-	-	-
Flavone *O*-sulfates	-	-	2	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-
Flavone *O*-glycoside sulfate	-	-	1	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-

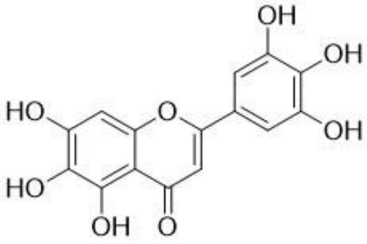
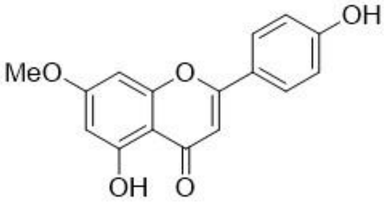
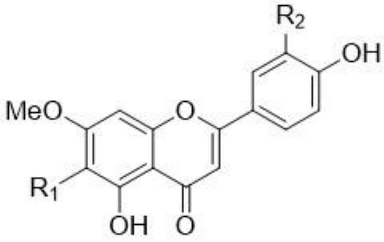
Table 4 – Marine flavones occurrence and biological activities.

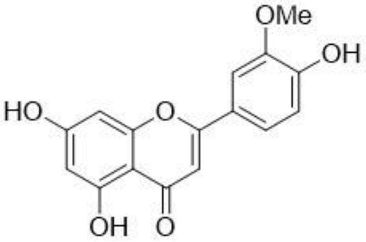
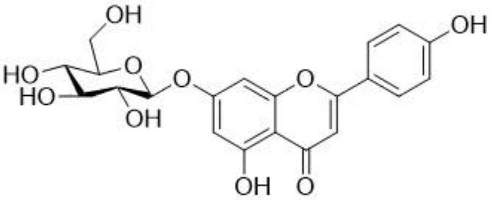
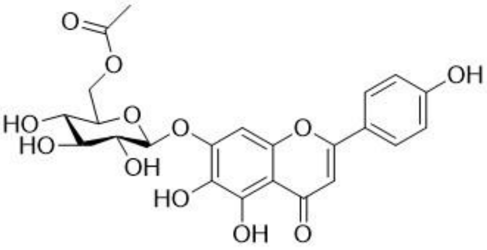
Flavones	**Organism species (Family)**	**Biological activities**	**Ref**
 R_1 = H; R_2 = H; Apigenin-7-sulfate (**1**) R_1 = OH; R_2 = H; Luteolin-7-sulfate (**2**) R_1 = OH; R_2 = Me; Diosmetin-7-sulfate (**3**) R_1 = OMe; R_2 = H; Chrysoeriol-7-sulfate (**4**) R_1 = OSO_3^-; R_2 = H; Luteolin-7,3'-disulfate (**5**)	Angiosperm *Zostera marina* (Zosteraceae)	-	(Harborne et al., 1976)
 6-hydroxyluteolin (**6**)	Seagrass *Phyllospadix japonica* (Zosteraceae)	-	(Takagi et al., 1979)
 Luteolin-5-methylether (**7**)	Seagrass *Phyllospadix japonica* (Zosteraceae)	-	(Takagi et al., 1979)

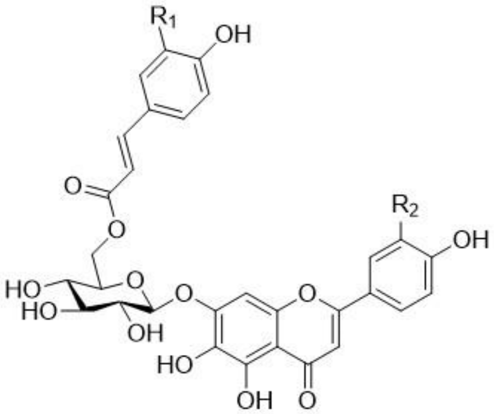
 Hispidulin (**8**)	Seagrass *Phyllospadix japonica* (Zosteraceae)	-	(Takagi et al., 1979)
	Seagrass *Phyllospadix iwatensis* (Cymodoceaceae)	-	(Takagi et al., 1980)
 R = H; Acacetin-5-methylether (**9**) R = OH; Luteolin-5,4’-dimethylether (**10**)	Seagrass *Phyllospadix japonica* (Zosteraceae)	-	(Takagi et al., 1979)
 R_1 = OMe; R_2 = H; Jaceosidin (**11**) R_1 = H; R_2 = Me; Pectolinarigenin (**12**) R_1 = OH; R_2 = Me; 5,7,3’-trihydroxy-6,4’-dimethoxyflavone (**13**)	Seagrass *Phyllospadix japonica* (Zosteraceae)	-	(Takagi et al., 1979)
 Phyllospadine (**14**)	Seagrass *Phyllospadix iwatensis* (Cymodoceaceae)	-	(Takagi et al., 1980)

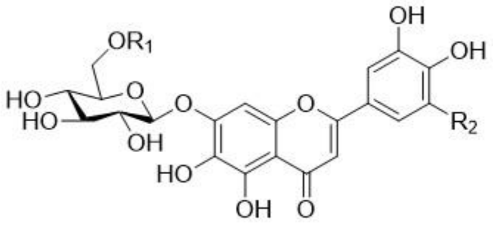
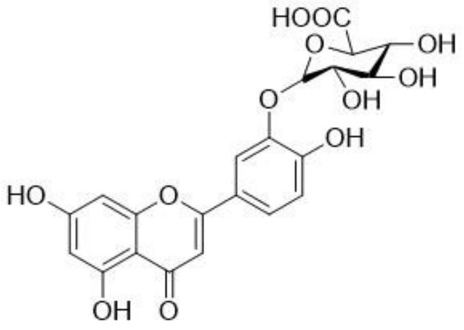
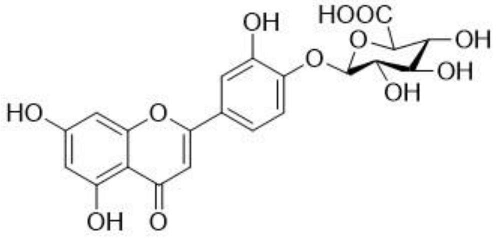
 Luteolin-7-*O*-*β*-D-glucopyranosyl-2”-sulfate (Thalassiolin A) (**15**)	Seagrass *Thalassia testudinum* (Hydrocharitaceae)	Antifouling activity	(Jensen et al., 1998)
 R = OMe; Chrysoeriol-7-*O*-*β*-D-glucopyranosyl-2”-sulfate (Thalassiolin B) (**16**) R = H; Apigenin-7-*O*-*β*-D-glucopyranosyl-2”-sulfate (Thalassiolin C) (**17**)	Seagrass *Thalassia testudinum* (Hydrocharitaceae)	Antimicrobial activity	(Rowley et al., 2002)
 Apigenin (**18**)	Mangrove *Avicennia marina* *(*Avicenniaceae)	Antioxidant activity	(Feng et al., 2006)
	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)
	Seagrass *Enhalus acoroides* (Hydrocharitaceae)	Antifeedant activity	(Qi et al., 2008)

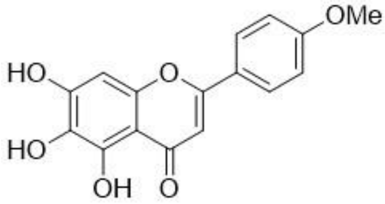
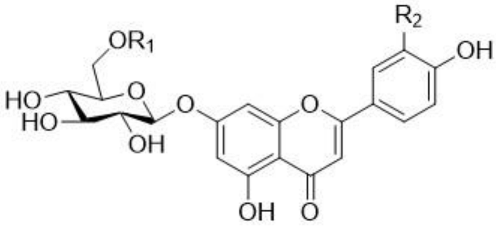
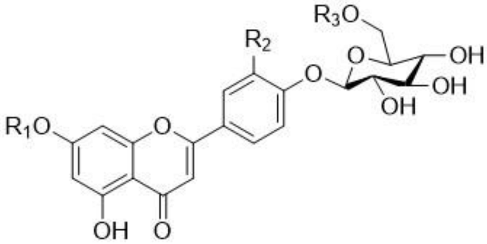
	Mollusk *Syphonota geographica* (Aplysiidae)	-	(Mollo et al., 2008)
	Halophyte *Juncus acutus* (Juncaceae)	Antioxidant activity	(Behery et al., 2012)
 5,3',4'-trihydroxy-7-methoxyflavone (**19**)	Mangrove *Avicennia marina* *(*Avicenniaceae)	Antioxidant activity	(Feng et al., 2006)
 R_1 = H; R_2 = H; 5,4'-dihydroxy-7,3'-dimethoxyflavone (**20**) R_1 = H; R_2 = OMe; 5,4'-dihydroxy-7,3',5'-trimethoxyflavone (**21**)	Mangrove *Avicennia marina* *(*Avicenniaceae)	-	(Feng et al., 2006)
 Luteolin (**22**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)

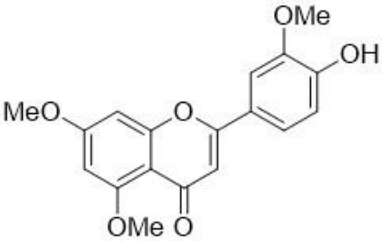
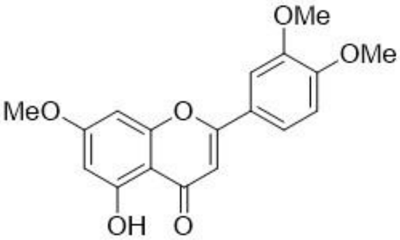
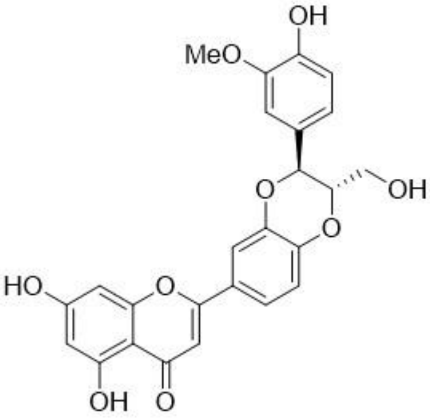
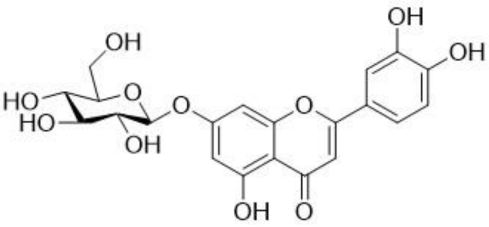
	Seagrass *Enhalus acoroides* (Hydrocharitaceae)	Antifouling, antimicrobial, and antifeedant activities	(Qi et al., 2008)
	Halophyte *Juncus acutus* (Juncaceae)	Antioxidant activity	(Behery et al., 2012)
 3',5'-dihydroxyscutellarein (**23**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)
 Genkwanin (**24**)	Mollusk *Syphonota geographica* (Aplysiidae)	-	(Mollo et al., 2008)
 R_1 = OH; R_2 = H; Ladanetin (**25**) R_1 = OH; R_2 = OH; Pedalitin (**26**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)

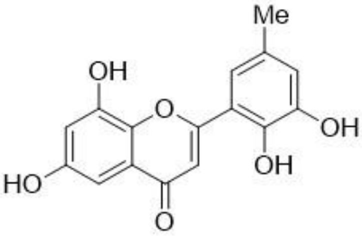
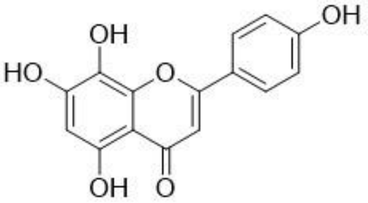
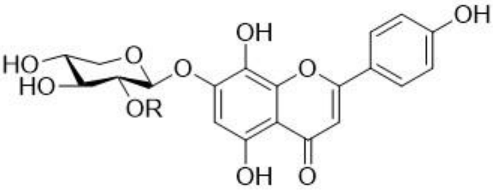
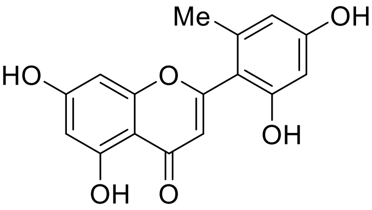
 Chrysoeriol (**27**)	Mollusk *Syphonota geographica* (Aplysiidae)	-	(Mollo et al., 2008)
	Halophyte *Juncus acutus* (Juncaceae)	Antioxidant activity	(Behery et al., 2012)
 Scutellarein-7-*O*-*β*-glucopyranoside (**28**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)
 6-hydroxyapigenin-7-*O*-(6”-*O*-acetyl)-*β*-glucopyranoside (**29**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)

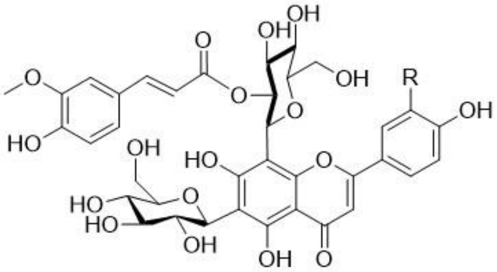
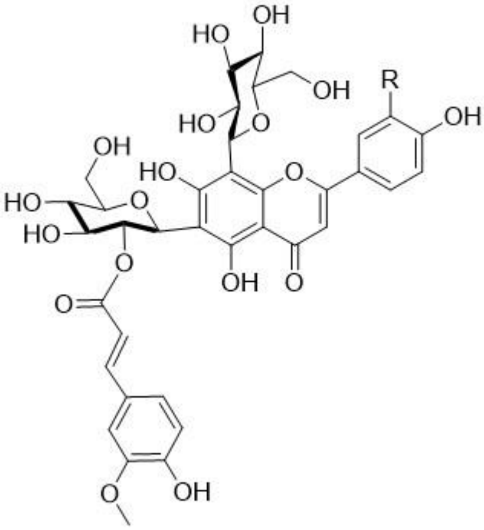
 R_1 = OH; R_2 = H; 6-hydroxyapigenin-7-*O*-(6”-*O*-[E]-caffeoyl)-*β*-glucopyranoside (**30**) R_1 = H; R_2 = H; 6-hydroxyapigenin-7-*O*-(6”-*O*-[E]-coumaroyl)-*β*-glucopyranoside (**31**) R_1 = H; R_2 = OH; 6-hydroxyluteolin-7-*O*-(6”-*O*-[E]-coumaroyl)-*β*-glucopyranoside (**32**) R_1 = OH; R_2 = OH; Spicoside (**33**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)

 R_1 = H; R_2 = H; 6-hydroxyluteolin-7-*O*-*β*-glucopyranoside (**34**) R_1 = Acetyl; R_2 = H; 6-hydroxyluteolin-7-*O*-(6”-*O*-acetyl)-*β*-glucopyranoside (**35**) R_1 = H; R_2 = OH; 5,6,7,3’,4’,5’-hexahydroxyflavone-7-*O*-*β*-glucopyranoside (**36**) R_1 = Acetyl; R_2 = OH; 5,6,7,3’,4’,5’-hexahydroxyflavone-7-*O*-(6”-*O*-acetyl)-*β*-glucopyranoside (**37**)	Angiosperm *Halophila johnsonii* (Hydrocharitaceae)	-	(Meng et al., 2008)
 Luteolin-3’-glucuronide (**38**)	Seagrass *Enhalus acoroides* (Hydrocharitaceae)	-	(Qi et al., 2008)
 Luteolin-4’-glucuronide (**39**)	Seagrass *Enhalus acoroides* (Hydrocharitaceae)	Antifouling, antimicrobial, and antifeedant activities	(Qi et al., 2008)

 Scutellarein-4’-methylether (**40**)	Algae *Osmundea pinnatifida* (Rhodomelaceae)	-	(Sabina et al., 2009)
 R_1 = H; R_2 = H Apigenin-7-*O*-*β*-glucopyranoside (**41**) R_1 = Malonyl; R_2 = H Apigenin-7-*O*-*β*-(6”-*O*-malonyl)-glucopyranoside (**42**) R_1 = H; R_2 = OMe; Chrysoeriol-7-*O*-*β*-glucopyranoside (**43**)	Seagrass *Halophila stipulacea* (Hydrocharitaceae	-	(Bitam et al., 2010)
 R_1 = H; R_2 = H; R_3 = H; Apigenin-4’-*O*-*β*-glucopyranoside (**44**) R_1 = H; R_2 = OMe; R_3 = H;Chrysoeriol-4’-*O*-*β*-glucopyranoside (**45**) R_1 = Me; R_2 = H; R_3 = H;Genkwanin-4’-*O*-*β*-glucopyranoside (**46**) R_1 = Me; R_2 = H; R_3 = Malonyl; Genkwanin-4’-*O*-*β*-(6”-*O*-malonyl)-glucopyranoside (**47**)	Seagrass *Halophila stipulacea* (Hydrocharitaceae)	-	(Bitam et al., 2010)

 4'-hydroxy-5,7,3'-trimethoxyflavone (**48**)	Seagrass *Thalassia hemprichii* (Hydrocharitaceae)	-	(Qi et al., 2012)
 5-hydroxy-7,3',4'-trimethoxyflavone (**49**)	Seagrass *Thalassia hemprichii* (Hydrocharitaceae)	-	(Qi et al., 2012)
 Hydnocarpin (**50**)	Halophyte *Juncus acutus* (Juncaceae)	Antioxidant activity	(Behery et al., 2012)
 Luteolin-7-*O*-*β*-D-glucoside (**51**)	Halophyte *Juncus acutus* (Juncaceae)	Antioxidant activity	(Behery et al., 2012)

 6,8,5’,6’-tetrahydroxy-3’-methylflavone (**52**)	Coral *Dichotella gemmacea* (Ellisellidae)	Antifouling activity	(Bao et al., 2013)
 Isoscutellarein (**53**)	Seagrass *Thalassia hemprichii* (Hydrocharitaceae)	Antimicrobial activity	(Hawas, 2014)
 R = H; Isoscutellarein-7-*O*-xylopyranoside (**54**) R = SO_3^-; Isoscutellarein-7-*O*-*β*-xyloside-2’’-sulfate (**55**)	Seagrass *Thalassia hemprichii* (Hydrocharitaceae)	Antimicrobial activity	(Hawas, 2014)
 Penimethavone (**56**)	Fungus *Penicillium Chrysogenum* (Trichocomaceae)	Antitumor activity	(Hou et al., 2016)

 R = H Apigenin 6-*C*-*β*-D-glucopyranoside 8-*C*-*β*-D-(2-*O*-feruloyl)-glucopyranoside (**57**) R = OH Luteolin 6-*C*-*β*-D-glucopyranoside 8-*C*-*β*-D-(2-*O*-feruloyl)-glucopyranoside (**58**)	Halophyte *Spergularia marina* (Caryophyllaceae)	-	(Cho et al., 2016)
 R = H Apigenin 6-*C*-*β*-D-(2-*O*-feruloyl)-glucopyranoside 8-*C*-*β*-D-glucopyranoside (**59**) R = OH Luteolin 6-*C*-*β*-D-(2-*O*-feruloyl)-glucopyranoside 8-*C*-*β*-D-glucopyranoside (**60**)	Halophyte *Spergularia marina* (Caryophyllaceae)	-	(Cho et al., 2016)

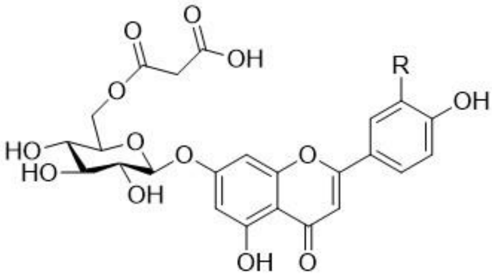
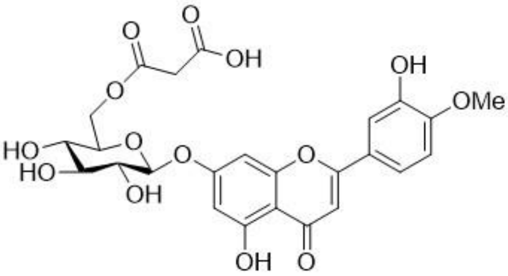
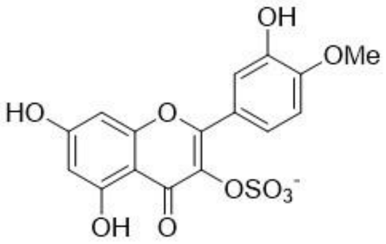
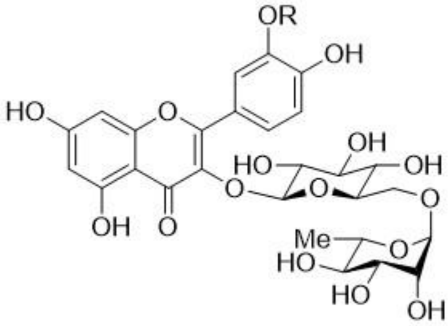
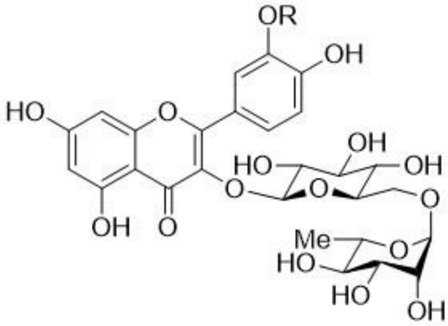
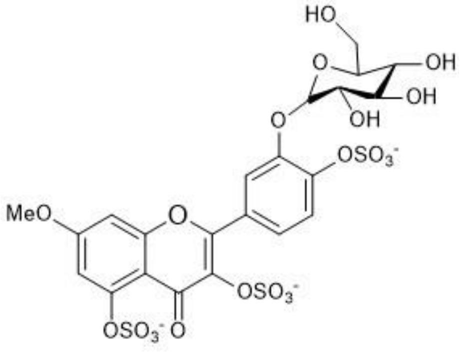
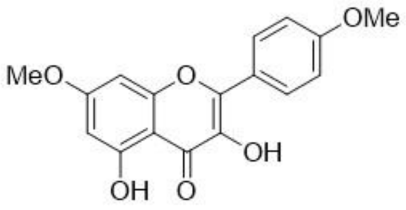
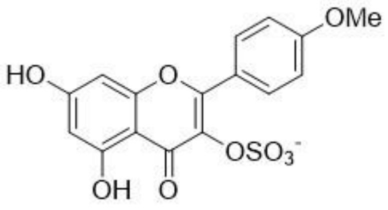
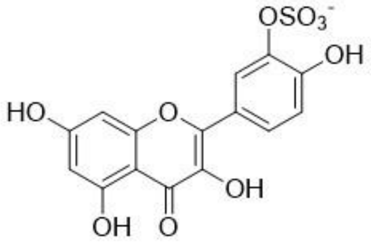
 R = OH Luteolin-7-*O*-*β*-(6''-malonyl)glucoside (**61**) R = H Apigenin-7-*O*-*β*-(6''-malonyl)glucoside (**62**) R = OMe Chrysoeriol-7-*O*-*β*-(6''-malonyl)glucoside (**63**)	Seagrass *Zostera marina* (Zosteraceae)	-	(Enerstvedt et al., 2016)
 Diosmetin-7-*O*-*β*-(6''-malonyl)glucoside (**64**)	Seagrass *Zostera marina* (Zosteraceae)	-	(Enerstvedt et al., 2016)

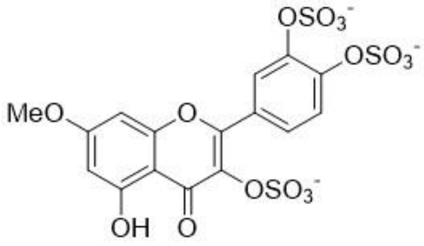
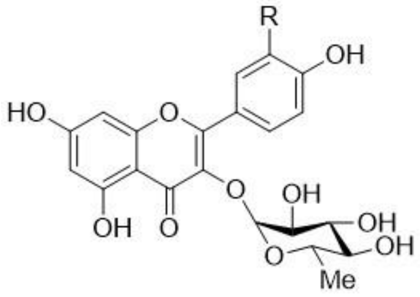
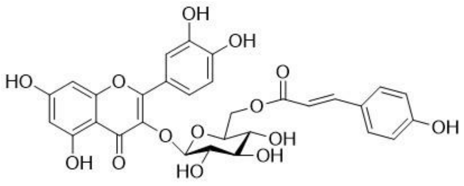
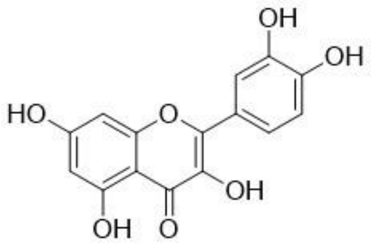
Table 5 – Frequency and substitution pattern of hydroxyl and methoxy groups among marine flavonols.

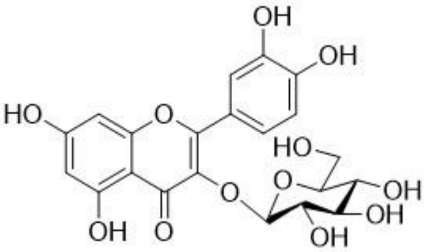
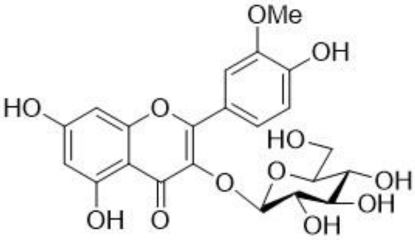
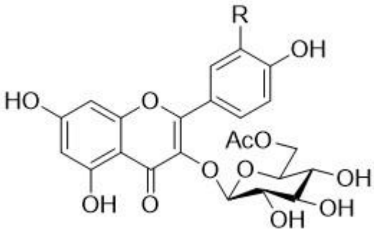
	7	5,7	5,4’	5,6,4’	5,7,3’	5,7,4’	5,8,4’	5,3’,4’	7,8,4’	5,6,7,4’	5,6,3’,4’	5,7,8,2’	5,7,8,5’	5,7,3’,4'	5,6,7,3’,4’	5,6,3’,4’,5’	5,7,2’,4’,6’	5,7,3’,4’,5’	6,8,3’,5’,6’	5,6,7,3’,4’,5’
Flavonol aglycones	-	-	-	-	-	2	-	-	-	-	-	2	-	1	-	-	-	-	-	-
Flavonol *O*-glycosides	1	-	-	-	-	3	-	-	-	-	-	-	-	6	-	-	-	-	-	-
Flavonol *O*-sulfates	1	1	-	-	-	2	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Flavonols *O*-glycoside sulfates	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

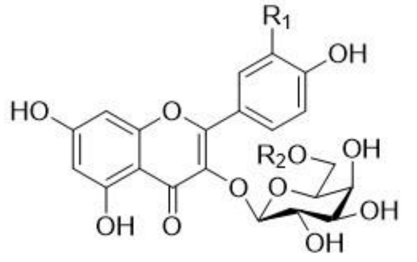
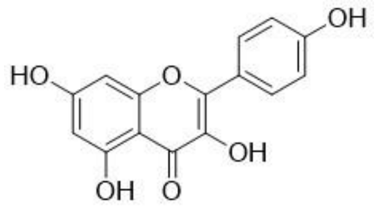
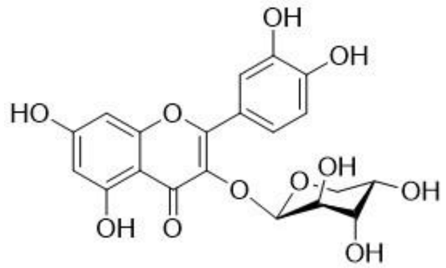
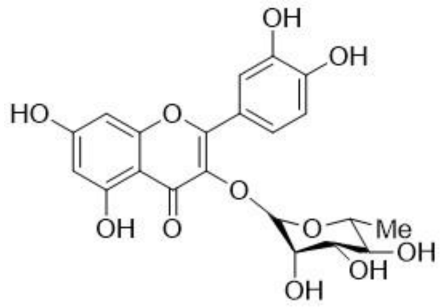
Table 6 - Marine flavonols occurrence and biological activities.

Flavonol	**Organism species (Family)**	**Biological Activities**	**Ref**
 Tamarixetin-3-sulfate (**65**)	Angiosperm *Tamarix laxa* (Tamaricaceae)	-	(Utkin, 1966)
	Halophyte *Tamarix gallica* (Tamaricaceae)	-	(Tomás-Barberán et al., 1990)
 R = Me; Isorhamnetin-3-*O*-rutinoside (**66**)	Halophyte *Batis marítima* (Bataceae)	-	(Utkin, 1966)
 R = H; Quercetin-3-*O*-rutinoside (**67**)	Halophyte *Apocynum venetum* (Apocynaceae)	-	(Kong et al., 2014)

 Rhamnetin-3,5,4’-*O*-trisulfate-3’-*O*-β-D-glucuronopyranosyl (**68**)	Angiosperm *Tamarix aphylla* (Tamaricaceae)	-	(Saleh et al., 1975)
 Kaempferol-7,4’-dimethylether (**69**)	Halophyte *Tamarix aphylla* (Tamaricaceae)	-	(El Ansari et al., 1976)
 Kaempferol 4’-methyl ether 3-sulfate (**70**)	Halophyte *Tamarix gallica* (Tamaricaceae)	-	(Tomás-Barberán et al., 1990)
 Quercetin-3’-sulfate (**71**)	Angiosperm *Hypericum elodes* (Guttiferae)	-	(Seabra et al., 1991)

 Rhamnetin-3,3’,4’-trisulfate (**72**)	Angiosperm *Tamarix amplexicaulis* (Tamaricaceae)	-	(Barakat, 1998)
 R = H; Kaempferol-3-*O*-*α*-L-fucopyranoside (Acanthophorin A) (**73**) R = OH; Quercetin-3-*O*-*α*-L-fucopyranoside (Acanthophorin B) (**74**)	Algae *Acanthophora spicifera* (Rhodomelaceae)	-	(Zeng et al., 2001)
 Tiliroside (**75**)	Algae *Acanthophora spicifera* (Rhodomelaceae)	-	(Zeng et al., 2001)
 Quercetin (**76**)	Algae *Acanthophora spicifera* (Rhodomelaceae)	-	(Zeng et al., 2001)
	Halophyte *Apocynum venetum* (Apocynaceae)	Antifouling activity	(Kong et al., 2014)

	Mangrove *Pelliciera rhizophorae* (Pellicieraceae)	-	(Park et al., 2015)
 Quercetin-3-*O*-*β*-D-glucoside (**77**)	Halophyte *Salicornia herbacea* (Amaranthaceae)	Antioxidant and antitumor activities	(Kong et al., 2008)
	Halophyte *Apocynum venetum* (Apocynaceae)	-	(Kong et al., 2014)
	Mangrove *Pelliciera rhizophorae* (Pellicieraceae)	-	(Park et al., 2015)
 Isorhamnetin-3-*O*-*β*-D-glucoside (**78**)	Halophyte *Salicornia herbacea* (Amaranthaceae)	Antioxidant and antitumor activities	(Kong et al., 2008)
 R = H; Kaempferol-3-*O*-(6”-O-acetyl)-*β*-D-glucopyranoside (**79**) R = OH; Quercetin-3-*O*-(6”-O-acetyl)-*β*-D-glucopyranoside (**80**)	Halophyte *Apocynum venetum* (Apocynaceae)	-	(Kong et al., 2014)

 R_1 = H; R_2 = Ac; Kaempferol-3-*O*-(6”-O-acetyl)-*β*-D-galactopyranoside (**81**) R_1 = OH; R_2 = H; Quercetin-3-*O*-galactoside (**82**)	Halophyte *Apocynum venetum* (Apocynaceae)	Antioxidant and antitumor activities	(Kong et al., 2014)
 Kaempferol (**83**)	Halophyte *Apocynum venetum* (Apocynaceae)	Antifouling activity	(Kong et al., 2014)
	Mangrove *Pelliciera rhizophorae* (Pellicieraceae)	-	(Park et al., 2015)
 Quercetin-3-*O*-*α*-arabinoside (**84**)	Mangrove *Laguncularia racemosa* (Combretaceae)	Anticoagulant activity	(Rodrigues et al., 2015)
 Quercetin-3-*O*-*α*-rhamnoside (**85**)	Mangrove *Laguncularia racemosa* (Combretaceae)	Anticoagulant activity	(Rodrigues et al., 2015)

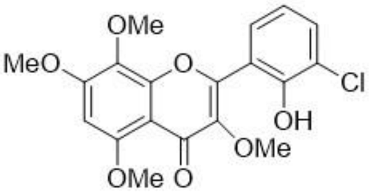
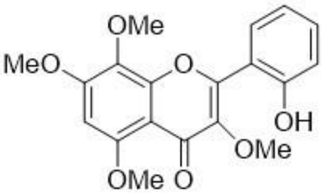
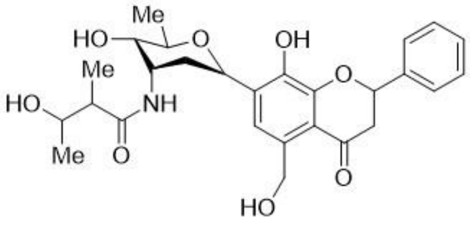
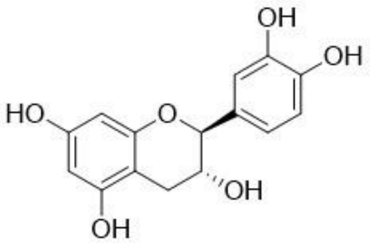
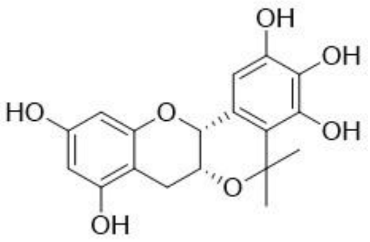
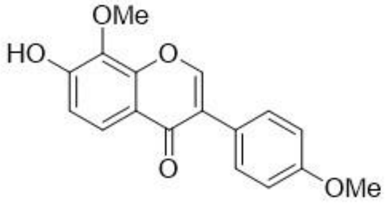
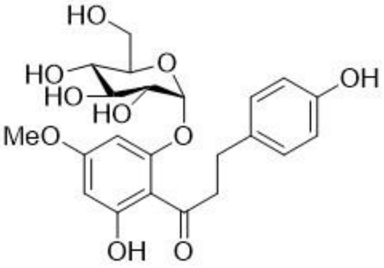
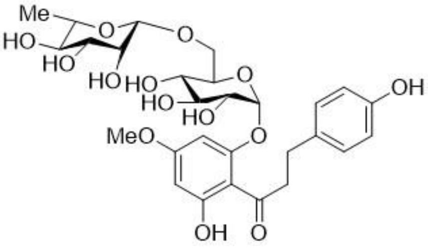
 Aspergivone A (**86**)	Fungus *Aspergillus candidus* (Trichocomaceae)	-	(Ma et al., 2017)
 Aspergivone B (**87**)	Fungus *Aspergillus candidus* (Trichocomaceae)	Antidiabetic activity	(Ma et al., 2017)

Table 7– Marine flavanones, flavan-3-ols, isoflavones, and dihydrochalcones occurrence and biological activities.

Flavonoids	Organism species (Family)	Biological Activities	Ref
 Actinoflavoside (**88**)	Bacterium *Streptomyces sp.* (Streptomycetaceae)	Antimicrobial activity	(Jiang et al., 1997)
 (-)-Catechin (**89**)	Algae *Acanthophora spicifera* (Rhodomelaceae)	-	(Zeng et al., 2001)
	Halophyte *Apocynum venetum* (Apocynaceae)	Antifouling activity	(Kong et al., 2014)
 Plumbocatechin A (**90**)	Halophyte *Apocynum venetum* (Apocynaceae)	-	(Kong et al., 2014)

 8-*O*-methylretusin (**91**)	Halophyte *Apocynum venetum* (Apocynaceae)	Antifouling activity	(Kong et al., 2014)
 Asebotin (**92**)	Seagrass *Thalassodendrin ciliatum* (Cymodoceaceae)	Antimicrobial activity	(Mohammed et al., 2014)
 Thalassodendrone (**93**)	Seagrass *Thalassodendrin ciliatum* (Cymodoceaceae)	Antimicrobial activity	(Mohammed et al., 2014)