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SEAWEEDS FROM THE PORTUGUESE COAST: CHEMISTRY, ANTIMICROBIAL AND ANTI-INFLAMMATORY CAPACITY

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Phytochemistry and Pharmacognosy Speciality

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“When we least expect it, we find what we have looked for during our entire life.”

To my parents and brother
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AUTHOR’S DECLARATION

The author declares that she has actively participated in the collection and study of the material included on all works and written all the manuscripts included in this dissertation, with the collaboration of other coauthors.
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RESUMO
RESUMO

Os oceanos compreendem uma imensa diversidade de organismos vivos, ainda pouco explorados quando comparados com os organismos terrestres. As algas são parte dessa diversidade, constituindo um dos mais importantes grupos de organismos, tanto em número como em variedade de espécies. Atendendo à pesquisa constante de compostos bioativos, as algas atraíram a atenção dos cientistas e tornaram-se objeto de muitos estudos nas últimas décadas. Portugal tem uma localização geográfica privilegiada, com uma vasta área costeira e uma gama de temperatura e exposição solar propícias ao desenvolvimento de várias espécies de algas. Com o objetivo de valorizar este recurso natural, dezoito espécies de algas colhidas na costa oeste Portuguesa pertencentes a três filos diferentes (Chlorophyta, Rhodophyta e Phaeophyta), foram exploradas no que respeita à sua composição química e atividade biológica.

O perfil de esteróis das amostras foi analisado por HPLC-DAD, permitindo a identificação e quantificação de 7 compostos: desmosterol, ergosterol, fucosterol, colesterol, campesterol, estigmasterol e β-sitosterol. As espécies pertencentes aos filos Chlorophyta e Phaeophyta caracterizaram-se pela presença maioritária de esteróis C_{29}, sendo o isofucosterol/fucosterol os principais compostos, respectivamente, enquanto as espécies do filo Rhodophyta se caracterizaram pela sua abundância em colesterol. As espécies dos filos Chlorophyta e Phaeophyta apresentaram o maior teor de esteróis, sendo a alga castanha *Cystoseira tamariscifolia* Hudson Papenfuss a espécie mais rica. Considerando os efeitos benéficos dos esteróis para a saúde humana, e tendo em conta o perfil qualitativo e quantitativo apresentado pelas espécies em estudo, as pertencentes aos filos Chlorophyta e Phaeophyta revelaram-se mais promissoras.

As espécies de algas de Phaeophyta foram analisadas relativamente ao seu teor total em florotaninos, determinado num extrato purificado pelo método específico do dimetoxibenzenaldeído. A espécie *Fucus spiralis* Linnaeus apresentou a maior quantidade destes compostos, seguida pelas espécies do género *Cystoseira*. Foram avaliadas as atividades anti-inflamatória, antimicrobiana e a capacidade de sequestro do óxido nítrico (NO) dos extratos purificados de florotaninos. A ação anti-inflamatória foi estudada através da avaliação da capacidade dos extratos para reduzir o NO produzido pela linha celular de macrófagos RAW 264.7. A espécie *C. tamariscifolia* apresentou a melhor atividade, sem toxicidade nas concentrações testadas. A atividade sequestrante para o NO foi avaliada também num sistema não celular, tendo os melhores resultados sido obtidos com o extrato de *F. spiralis*. A atividade antimicrobiana dos extratos foi avaliada relativamente a uma vasta gama de bactérias e fungos patogênicos, sendo mais ativos...
contra bactérias Gram\(^+\) e dermatófitos, com destaque para as espécies *F. spiralis* e *Cystoseira nodicaulis* (Withering) M. Roberts.

O perfil de florotaninos das algas mais promissoras, ou seja, aquelas com maiores quantidades de florotaninos e melhores atividades biológicas, foi analisado por HPLC-DAD-ESI/MS\(^n\). Foram caracterizados 22 florotaninos nas espécies estudadas, pertencentes às classes ecol e fucofloroetol: 8 em *C. nodicaulis*, 2 em *C. tamariscifolia*, 4 em *Cystoseira usneoides* (Linnaeus) M. Roberts e 8 em *F. spiralis*. A capacidade dos extratos de florotaninos destas espécies para sequestrar o radical anião superóxido, para inibir a peroxidação lipídica e para prevenir a degradação do ácido hialurônico, por meio da inibição da hialuronidase, foi determinada. Todas as espécies estudadas apresentaram resultados promissores, sendo a *F. spiralis* a mais ativa.

Tendo em conta o aparecimento crescente de resistências a antifúngicos, a atividade antifúngica de extratos purificados de florotaninos foi aprofundada, relativamente a uma variedade mais alargada de leveduras e dermatófitos, demonstrando atividade fungistática contra leveduras e fungicida contra dermatófitos. O mecanismo de ação dos extratos purificados de florotaninos foi também avaliado, usando a levedura *Candida albicans* e o dermatófito *Trichophyton rubrum* como modelos, e baseou-se no seu efeito sobre os alvos mais comuns dos fungos. Embora os florotaninos não apresentem um efeito claro sobre os componentes da membrana e parede celular dos fungos, o seu efeito sobre a rede metabólica dos microorganismos é notório. Adicionalmente, a espécie *F. spiralis* apresentou resultados promissores na inibição do fator de virulência de *C. albicans*.

Dado o potencial químico e biológico da espécie *F. spiralis*, o seu estudo foi aprofundado e prosseguiu-se com o isolamento de compostos. Dois monogalactosil diacilgliceróis (MGDGs) e um monoacilglicerol foram isolados por métodos cromatográficos e as suas estruturas foram elucidadas por meio de espectroscopia (RMN e MS) e por comparação com a literatura. O monoacilglicerol, composto por glicerol ligado a ácido oleico (C18:1 \(\Delta 9\)) e os MGDGs, compostos por uma porção de glicerol ligado a uma unidade de galactose e de ácido eicosapentaenoico (C20:5 \(\Delta 3\)) combinado com o ácido linolénico (C18:3 \(\Delta 3\)) ou ácido octadecatetraenoico (C18:4 \(\Delta 3\)), respetivamente, foram testados quanto à sua atividade citotóxica e anti-inflamatória em macrófagos RAW 264.7, apresentando capacidade para inibir a produção de NO em concentrações não citotóxicas.

As algas estudadas apresentaram características biológicas interessantes, sendo as espécies do género *Cystoseira* e o *F. spiralis* particularmente promissoras para o possível desenvolvimento de formulações farmacêuticas e de alimentos funcionais.

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Resumo
Palavras-chave: Atividade anti-inflamatória; atividade antimicrobiana; atividade antioxidante; esteróis; florotaninos; glicerolípidos; macroalgas.
ABSTRACT
ABSTRACT

Oceans comprise an immense diversity of living organisms, still underexplored if compared with terrestrial ones. Seaweeds are part of this diversity, constituting one of the most important groups of organisms, in both number and variety of species. Considering the constant search for new bioactive compounds, seaweeds have attracted scientists’ attention and became the subject of many studies in the last decades. Portugal has a privileged geographical location, with a wide coastal area and a range of temperature and sun exposition propitious to the development of various species of seaweeds. With the aim of valuing this natural resource, seaweeds species belonging to three different phyla (Chlorophyta, Rhodophyta and Phaeophyta) were analyzed and explored for their chemical composition and biological activities.

The sterols profile of eighteen seaweeds was analyzed by HPLC-DAD, allowing the identification and quantification of 7 compounds: desmosterol, ergosterol, fucosterol, cholesterol, campesterol, stigmasterol and β-sitosterol. Species belonging to Chlorophyta and Phaeophyta contained mainly C_{29} sterols, isofucosterol/fucosterol being the major compounds, respectively, while the ones of Rhodophyta where characterized by their abundance in cholesterol. Chlorophyta and Phaeophyta presented the highest total sterols content, the brown seaweed *Cystoseira tamariscifolia* Hudson Papenfuss being the richest species. Considering the sterols health effects and the qualitative and quantitative profile presented by the studied seaweeds, Chlorophyta and Phaeophyta represent the most promising groups.

Phaeophyta species were analyzed for their total phlorotannins content by their quantification in a purified phlorotannins extract using the dimethoxybenzaldehyde specific assay. Of the studied species, *Fucus spiralis* Linnaeus presented the highest total phlorotannins amount, followed by the *Cystoseira* species. The anti-inflammatory, antimicrobial and NO scavenging activity of the purified extracts was analyzed. The anti-inflammatory capacity was studied by evaluating the capacity of the extracts to reduce the NO produced by RAW 264.7 macrophage cells, *C. tamariscifolia* presenting the best activity, with no citotoxicity under the tested concentrations. The NO scavenging activity was evaluated on a cell-free system, *F. spiralis* presenting the best results. The antimicrobial activity was evaluated over a wide range of pathogenic Gram^+^ and Gram^-^ bacteria and fungi. In a general way, phlorotannins extracts were more active against Gram^+^ bacteria and, among fungi, against dermatophytes, with emphasis on *F. spiralis* and *Cystoseira nodicaulis* (Withering) M. Roberts.
The phlorotannins profile of the most promising seaweeds, i.e., the ones with higher phlorotannins amounts and better biological activities, was analyzed by HPLC-DAD-ESI/MS. Twenty two different phlorotannins belonging to eckol and fucophlorethol main groups were characterized in the studied seaweeds: 8 in *C. nodicaulis*, 2 in *C. tamariscifolia*, 4 in *Cystoseira usneoides* (Linnaeus) M. Roberts and 8 in *F. spiralis*. The capacity of the phlorotannins extracts of these species to sequester superoxide anion radical, to inhibit lipid peroxidation and to prevent the degradation of hyaluronic acid, by the inhibition of hyaluronidase, was determined. All of the studied species presented promising results; nevertheless, *F. spiralis* was, by far, the most active species.

Taking into account the emergence of resistance to antifungal drugs, the antifungal activity of purified phlorotannins extracts was extended to a larger number of yeasts and dermatophytes, demonstrating fungistatic activity against yeast and fungicidal capacity against dermatophytes. The mechanism of action of purified phlorotannins extracts was also evaluated, using the yeast *Candida albicans* and the dermatophyte *Trichophyton rubrum* as models. The effect of purified phlorotannins extracts was evaluated over the most common fungal targets. Although phlorotannins did not present a clear effect on fungal cell membrane and cell wall, they clearly interacted with the microorganism’s metabolic network. Additionally, *F. spiralis* presented promising results on the inhibition of *C. albicans* virulence factor.

Considering the potential of the seaweed species *F. spiralis*, the studies were deepened and continued with the isolation of compounds. Two monogalactosyl diacylglycerols (MGDGs) and one monoacylglycerol were isolated by chromatographic methods and their structures were elucidated by spectroscopic means (NMR and MS) and by comparison with the literature. The monoacylglycerol was composed of a glycerol moiety linked to oleic acid (C18:1 Ω9) and the MGDGs contained a glycerol moiety linked to a galactose unit and eicosapentaenoic acid (C20:5 Ω3) combined with linolenic acid (C18:3 Ω3) or octadecatetraenoic acid (C18:4 Ω3), respectively. The isolated compounds were tested for their cytotoxic and anti-inflammatory activity in RAW 264.7 macrophage cells, presenting the capacity to inhibit NO production at non-cytotoxic concentrations.

The studied seaweeds demonstrated interesting nutritional and biological features, *Cystoseira* species and *F. spiralis* being particularly promising for the possible development of pharmaceutical formulations and functional food products.

**Keywords:** Anti-inflammatory activity; antimicrobial activity; antioxidant activity; glycerolipids; phlorotannins; sterols; seaweeds.
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