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EVALUATION OF THE ANTICANCER AND ANTI-INFLAMMATORY POTENTIAL OF THE BROWN SEAWEED *CYSTOSEIRA*

by

AHMAD A FARAG MANSUR

A thesis submitted to Plymouth University in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award. This work submitted for the research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment. This study was financed by the Ministry of Higher Education& Scientific Research /Libya.

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Ahmad A Farag Mansur

Evaluation of the anticancer and anti-inflammatory potential of the brown seaweed *Cystoseira*

Ahmad A Farag Mansur

ABSTRACT

Cystoseira is abundant in bioactive chemicals that have been emphasized for their pharmacological value as plentiful and typically safe alternatives, offering a viable technique for treating cancer and inflammatory diseases. However, evaluating the effect of seasonality and extraction methods on *Cystoseira* chemical composition and cytotoxicity activity are still not thoroughly investigated. This study investigates the effect of seasonal variations and extraction methods of the *Cystoseira* extracts on the chemical composition and cytotoxicity activity on human cancer cell lines. *C. tamariscifolia*, *C. crinita* and *C. compressa,* were collected from the UK and Libya and colourimetric techniques were used to measure the chemical components. The cell vitality was measured *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Trypan Blue (TB) assays. The results showed that both chemical composition and anticancer activity were subject to the seasonality and extraction method. The study also confirmed that the three *Cystoseira* spices demonstrated potent anticancer activity against human leukaemia HL60 and THP-1, prostate PC3 and lymphoma REC-1 cancer cell lines. In an effort to investigate the anti-proinflammatory activities of *Cystoseira* extracts, human macrophage-like cells were exposed to the crude extracts and fractions and the release of TNF-α and IL-1β was measured using the enzyme-linked immunosorbent assay (ELISA). The results reveal that the methanol extract of *C. tamariscifolia* and *C. crinita* extracts significantly inhibited the release of TNF-α and IL-1β. *C. crinita* methanolic extract was partitioned using HPLC, and the fractions were tested for their anti-TNF-α release effect. The result showed that the sup-fraction C1c from C. *crinita* had a significant anti-proinflammatory effect and the LC-MS analysis of the fraction showed that it contains fatty acids and mannitol. These data suggest that sampling times and extraction methods should be considered to maximise the possibility of finding novel bioactive compounds in *Cystoseira*. The study also suggests that *C. crinita* is a valuable source of anti-inflammatory agents. This should provide helpful information for medicinal chemists in their attempts to develop anticancer and immunomodulatory agents. However, more research on this topic is needed to understand *Cystoseira* bioactive compounds mechanisms of action.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

AUTHOR'S DECLARATION AND PUBLICATIONS iv

ABSTRACT v

TABLE OF CONTENTS vi

LIST OF FIGURES xi

LIST OF TABLES xv

LIST OF ABBREVIATIONS xiv

CHAPTER 1 1

GENERAL INTRODUCTION 1

1 Introduction 2

1.1 *Cystoseira* genus 2

1.1.1 *Cystoseira* genus distribution 4

1.1.2 Cultivation and harvesting 6

1.1.3 Seasonal and locational variation in bioactive compositions of *Cystoseira* 8

1.1.4 *Cystoseira* bioactive composition 11

1.2 Cancer diseases 16

1.2.1 The need for natural products for new anticancer drugs 18

1.2.2 Brown seaweed as a source of anticancer bioactive compounds 19

1.3 Inflammation 22

1.3.1 Inflammatory disease and the need for therapy 27

1.3.2 Brown seaweed as a source of anti-proinflammatory agents 31

1.4 Aims of the current study 37

CHAPTER 2 39

GENERAL MATERIALS AND METHODS

2	General materials and methods	40
2.1	Collection of *Cystoseira* spp.	40
2.2	Solvent extraction	40
2.3	Total polyphenol content (TPC)	40
2.4	Total flavonoid content (TFC)	40
2.5	Total polysaccharides content	41
2.6	Total protein	41
2.7	Cell line maintenance and culture	41
2.8	Cell vitality assay	41
2.9	Determining the cell vitality of cancer cells lines using Trypan Blue Stain on Automated Cell Counter	42
2.10	Fluorescent staining for HL60 and THP-1 cells	42
2.11	Heat-killing *Escherichia coli* strain K12	42
2.12	Limulus Amebocyte Lysate (LAL) endotoxin detection assay	42
2.13	THP-1 Cell differentiation using phorbol 12-myristate 13-acetate (PMA)	43
2.14	Macrophage-like cells stimulation by Lipopolysaccharides (LPS), Lipoteichoic acid (LTA) heat-killed and live *E. coli* K12 and exposition to the *Cystoseira* methanol extracts	43
2.15	Detection of *Cystoseira* immunomodulatory activity on TNF-α and IL-1β release from M1 macrophage-like using ELISA.	43
2.16	Bacteria culture preparation	44
2.17	Procedure for performing the disc diffusion test	44
2.18	*Cystoseira* crude extract profiling and fractionation by High-performance Liquid Chromatography (HLPC)	45
2.19	Initial HPLC fractionation of methanol extract of *Cystoseira* spp.	45
2.20	Statistical analysis	45

2.21 Sample preparation for LC/UV (DAD) and LC/ESI-MS analysis 45

2.22 LC/UV (DAD) analysis 46

2.23 LC-ESMS analysis 46

CHAPTER 3 48

THE BIOCHEMICAL COMPOSITION AND CYTOTOXIC ACTIVITY OF THE BROWN ALGA *CYSTOSEIRA* SPP. AGAINST CANCER CELL LINES

3.1 Introduction 49

3.2 Results 52

3.2.1 The biochemical composition of *Cystoseira crinita* and *Cystoseira compressa* 52

3.2.2 The cytotoxic activity of the Mediterranean brown alga *Cystoseira crinita* and C. *compressa* against cancer cell lines 54

3.2.3 Effects of extraction method and season on the biochemical composition of *Cystoseira tamariscifolia* extracts 57

3.2.4 Extraction method and seasonality impact the anticancer activity of *C. tamariscifolia*. 59

3.2.5 The suggested correlation between *Cystoseira tamariscifolia* polyphenols and the cytotoxic activity against three cancer cell lines 65

3.2.6 Methanol extracts of *Cystoseira* extract against cancer cells lines using Trypan Blue Stain on Automated Cell Counter 67

3.2.7 *Cystoseira tamariscifolia* methanol extracts exhibit apoptosis and nuclei fragmentation on HL60 and THP-1 cell lines 76

3.3 Discussion 78

CHAPTER 4 84

METHANOL EXTRACT OF BROWN ALGAE *CYSTOSEIRA* SPP. INHIBITS PRO-INFLAMMATORY RESPONSE IN STIMULATED HUMAN MACROPHAGE-LIKE CELLS

4.1 Introduction 85

4.2 Results 89

4.2.1 *Cystoseira* extracts suppress pro-inflammatory cytokines TNF-α and IL-1β release in Human LPS-stimulated macrophage-like cells 89

4.2.2 Effects of *Cystoseira* methanol extracts on lipoteichoic acid (LTA) induced TNF-α release 91

4.2.3 The cytotoxic effect of the methanol extracts of *Cystoseira* species on M1 macrophage-Like cells 92

4.2.4 Methanol extracts of *Cystoseira* spp exhibit anti- endotoxin activity 93

4.2.5 Effects of *Cystoseira* methanol extract on heat-killed and live *Escherichia coli* induced TNF-α and IL-1β release 97

4.2.6 The antibacterial activity of extracts of *Cystoseira* spp. 101

4.3 Discussion 103

CHAPTER 5 110

ISOLATION AND IDENTIFICATION OF ANTI-INFLAMMATORY COMPOUNDS FROM *CYSTOSEIRA* SPP.

5.1 Introduction 111

5.2 Results 113

5.2.1 An initial HPLC chromatographic profiling of methanol extract of *Cystoseira* spp. 113

5.2.2 The effects of *Cystoseira* spp. methanol extract fractions on LPS-induced TNF-α release 115

5.2.3 The effect of sup-C1 fractions of *Cystoseira crinita* on LPS-induced TNF-α in M1macrophage-like cells 117

5.2.4 The identification of anti-proinflammatory compounds in fraction C1c from *Cystoseira crinita* among the HPLC–DAD–ESI/MS (positive and negative ion mode) 120

5.3 Discussion 125

CHAPTER 6 131

GENERAL DISCUSSION

6.1 Discussion 132

6.2 Future work 141

REFERENCES 146

Appendices 188

LIST OF FIGURES

Chapter 1

Figure 1.1 Morphological features of the *Cystoseira* genus. ……………………….…………..….3

Figure 1.2 The map of *Cystoseira* distribution. …….……………………….……………………..5

Figure 1.3 Pictures of *Cystoseira* species; (A) *Cystoseira tamariscifolia* (Hudson) Papenfuss1950, (B) *Cystoseira crinita* Duby 1830 and (C) *Cystoseira compressa* (Esper) Gerloff and Nizamuddin 1975. …………………...…………………………...……..…6

Figure 1.4 The life cycle of *Cystoseira*. ……………………………………………..………..8

Figure 1.5 Macrophage activation during infection and inflammation………………………24

Figure 1.6 Cellular pro-inflammatory response pathways …………………...………………26

Chapter 3

Figure 3.1 Dose-response curves for cell vitality in cancer cell lines. HL60, THP-1, PC3 and REC-1 A288 cells were exposed for 72 h to crude extracts of *C. crinita*. Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3)………….. ……………………………………………………….……….55

Figure 3.2 Dose-response curves for cell vitality in cancer cell lines. HL60, THP-1, PC3 and REC-1 A288 cells were exposed for 72 h to crude extracts of *C. compressa.* Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3)………….. ……………….……………………………………….....…56

Figure 3.3 Dose-response curves for cell vitality in leukaemia cancer cells. HL60 cells were exposed for 72 h to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.3: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).. 61

Figure 3.4 Dose-response curves for cell vitality in prostate cancer cells. PC3 cells were exposed for 72 h to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.4: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3)……………….....................................…62

Figure 3.5 Dose-response curves for cell vitality in leukaemia cancer cells. THP-1 cells were exposed for 72 h to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.5: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).…………………………………………63

Figure 3.6 Dose-response curves for cell vitality in mantle cell lymphoma. REC-1 A288 cells were exposed for 72 h to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.6: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3)…….……………………………64

Figure 3.7 Scatter plots show relationships between the concentration of TPC extracted from *C. tamariscifolia* and IC_{50} values data from HL60 (A), THP-1(B) and PC3(C) cytotoxic assay. Graphs show a negative correlation between TPC concentration and IC_{50} values and therefore positive correlation between the concentration of TPC and the cytotoxicity effect of the extracts. ……………………..…….……………………….……………………………...66

Figure 3.8 Vitality assay via cell staining using the LIVE/DEAD /Cytotoxicity Kit. HL60 cell line exposed to *C. tamariscifolia, C crinita* and *C compressa* and incubated for 72h at 37 °C. Treated cell lines and control stained with diluted Trypan blue stain and calculated with the Countess II FL Automated Cell Counter instrument equipped with standard Light Cubes. The histogram and the picture show the counts of live cells in the population, which fluoresce green, and of dead cells, which fluoresce red... ……………………………………………...…..71

Figure 3.9 Vitality assay via cell staining using the LIVE/DEAD /Cytotoxicity Kit. THP-1 cell line exposed to *C. tamariscifolia*, *C. crinita* and *C. compressa* and incubated for 72h at 37 °C. Treated cell lines and control stained with diluted Trypan blue stain and calculated with the Countess II FL Automated Cell Counter instrument equipped with standard Light Cubes. The histogram and the picture show the counts of live cells in the population, which fluoresce green, and of dead cells, which fluoresce red..……………….……..…………………………..…75

Figure 3.10 Fluorescent staining of cancer cells nuclei by propidium iodide. Cancer cells (A) HL60 and (B) THP-1 were treated with %100 methanol extracts at the concentration of 150 μg mL-1 for 48h. Fragmented nuclei and apoptotic bodies were seen in the *C. tamariscifolia* extracts-treated cells (b) but not in the control treatment (a). Magnification 200×………..…77

Chapter 4

Figure 4.1 Effect of *Cystoseira* extracts on TNF-α and IL-1β release in LPS-stimulated macrophage-like cells for 20 h incubation. After 20 h incubation, TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and LPS (1 μg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** P<0.05 vs LPS alone. .. ..………………………………………………….…90

Figure 4.2 Effect of *Cystoseira* extracts on TNF-α release in LTA-stimulated macrophage-like cells. After 20 h incubation, TNF-α released into the culture medium were assayed by the ELISA method. No treatment control (-) and LTA alone (5 μg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** P<0.05 vs LTA alone…………………………………………………………………………………….91

Figure 4.3 Percentage of cell vitality in comparison to control (mean ± SD; n=3). M1 Macrophage-like cells were exposed for 20 h to 100% methanol crude extracts of *Cystoseira* species.. 92

Figure 4.4 Endotoxin detection in extracts and LPS with extracts using the endpoint chromogenic LAL assay. LPS (1 μg mL^{-1}) and the mixture of LPS and extracts *C. tamariscifolia* and *C. crinita* (1 mg mL^{-1}) were incubated with LAL and substrate. After the incubation period, the absorbance of the substrate was measured. The graph shows that no endotoxin contamination in extracts.. …..…94

Figure 4.5 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in LPS-stimulated macrophage-like cells. *Cystoseira* extracts were added together with 1 μg mL^{-1} LPS. After 20 h incubation, TNF-α and IL-1β produced and released into the culture medium were assayed

by the ELISA method. No treatment control (-) and LPS (1µg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** P<0.05 vs LPS alone……………………………………………………………………………………95

Figure 4.6 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in LPS-stimulated macrophage-like cells. *Cystoseira* extracts were added after 2 h of adding 1 µg mL^{-1} LPS. After 20 h incubation, TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and LPS (1 µg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. * P<0.05 vs LPS alone. ….………………..96

Figure 4.7 TNF-α release when M1 macrophage-like cells stimulated by heat-killed *E. coli* versus when stimulated by LPS. Cells were cultured for 20 h in the presence of increasing concentrations of both LPS and heat-killed *E. coli* bacteria. Data represent the mean value (mean ± SD) of the cytokine in pg mL^{-1} of cell culture supernatant for triplicate samples for each test treatment. ……………………………………………………………………...…..98

Figure 4.8 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in heat-killed *E. coli* k12-stimulated macrophage-like cells. After 20 h incubation, TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and heat-killed *E. coli* (10^{9} cell mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** P<0.05 and vs heat-killed *E. coli* alone..……………………..…99

Figure 4.9 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in live *E. coli* k12-stimulated macrophage-like cells after 20 h. TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and live *E. coli* (10^{9} cell mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** P<0.05 and vs Live *E. coli* alone..…………………………………......…100

Chapter 5

Figure 5.1 HPLC chromatogram of the methanol extracts of *C. tamariscifolia* (A) and *C. crinita*…………………………..……………………………………………………...……114

Figure 5.2 Effect of *C. tamariscifolia* (A) and *C. crinita* (B) methanol extract fractions on TNF-α in LPS-stimulated M1 macrophage-like cells. TNF-α released into the culture medium was assayed by the ELISA method. The data represent the mean ± SD of triplicate experiments. * P<0.05 vs LPS alone...……………………………………...……………….....…..…116

Figure 5.3 HPLC profile of the active anti-proinflammatory fraction C1 from C. *crinita* extract. ……………………..…………………………………………………………………..…118

Figure 5.4 Effect of *C. crinita* extracts fractions C1, C1a, C1b and C1c on TNF-α in LPS-stimulated M1 macrophage-like cells. TNF-α released into the culture medium was assayed by the ELISA method. The data represent the mean ± SD of triplicate experiments. * P<0.05 vs LPS alone.. 118

Figure 5.5 Percentage of cell vitality in comparison to control (mean ± SD; n=3). Macrophage-like cells were exposed to C1 fractions of *C. crinita* for 20 h.. ……………………………119

Figure 5.6 L.C./UV-Vis chromatogram (A) and 3-D DAD spectrum (B) of C1c fraction from *C. crinita*. ……….……………………….…………………………..………….………..121

Figure 5.7 LC/ESI–MS background subtracted base peak chromatogram of C1c fraction in both Positive Ion LC/ESI-MS Analysis (A) and Negative Ion LC/ESI-MS Analysis (B)…...122

Figure 5.8 Mass spectrum of fatty acids and mannitol appeared in the active proinflammatory fraction C1c from *C. crinita*. (A) oleic acid, (B) nonanedioic acid, (C) undecanedioc acid, (D) palmitic acid and (E) mannitol..…………………………….………....……………...….…..…124

LIST OF TABLES

Table 1.1 *Cystoseira* biochemical compounds exhibiting biological activity……….……..13

Table 3.1 The bioactive compound compositions of the brown seaweed *Cystoseira* spp. Collected in the U.K. and Libyan coasts. *D.W.:* dry weight. Total polyphenol Content: milligram phloroglucinol acid equivalent per gram dry weight; Total flavonoid Content: milligram Quercetin equivalent per gram dry weight. Total polysaccharide: milligram Glucose equivalent per gram dry weight; Total protein: milligram bovine serum albumin equivalent per gram dry weight. Values are presented as mean ± S.D. (n = 3)... ……………………………..…………………………………………………………..…..49

Table 3.2 The bioactive compositions of the brown seaweed *C. tamariscifolia*. Collected from Hannafore Point, The south-western coast of Britain. *DW:* dry weight. Total polyphenol Content: milligram phloroglucinol acid equivalent per gram dry weight; Total flavonoid Content: milligram Quercetin equivalent per gram dry weight. Total polysaccharide: milligram Glucose equivalent per gram dry weight; Total protein: milligram bovine serum albumin equivalent per gram dry weight. Values are presented as mean ± S.D. (n = 3)... …..……………………………………………………………………………………...54

Table 3.3 IC_{50} values (µg mL^{-1}) for extracts on HL60, PC3 and THP-1 cell lines. Cells were exposed for 72 h to crude extracts of *Cystoseira tamariscifolia* collected in all seasons. IC_{50} values were calculated by sigmoidal dose-response of the data using SigmaPlot v. 13.0……………………………………….…………………….…………………56

Table 4.1 The antibacterial activity of methanolic extracts of *Cystoseira* spp using disk diffusion assay. Average diameter (±SE) of inhibition zone of *C. tamariscifolia*, *C. crinita* and *C. compressa* methanol extracts at concentrations (1, 3, 6, 10 and 100 mg mL^{-1}) against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* strain K12 using disc diffusion method and gentamicin as a standard antibiotic...………………….……….……...97

Table 5.1 Identification of the active anti proinflammatory compounds Mannitol and fatty acids in C1c fraction of *Cystoseira crinita* observed by HILIC–ESI–MS, as positive ions mode and negative ion mode showing their molecular mass, retention time and molecular formula………………………………………………………………………………...116

LIST OF ABBREVIATIONS

CAK	Cdk-Activating Kinase or
CPC	Centrifugal Partition Chromatography
DW	Dry Weight
HPLC	High-Performance Liquid Chromatography
HILIC/ES I-MS	Hydrophilic liquid chromatography-electrospray ionization mass spectrometry
IL-1β	Interleukin-1 Beta
IARC	International Agency for Research on Cancer
IFN-γ	Interferon-gamma
JAK	Janus kinase
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-NMR	Liquid Chromatography-Nuclear Magnetic Resonance
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acid
MAPK	Mitogen-Activated Protein Kinase
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear Magnetic Resonance Spectrometry
PGE	Phloroglucinol Equivalents
PI3K	phosphatidylinositol-3-kinase
PMA	Phorbol 12-Myristate 13-Acetate
PUFAs	Polyunsaturated Fatty Acids
SD	Standard Deviation
SEM	Standard Error of The Mean
STAT3	Signal Transducer and Activator of Transcription 3
TFC	Total Flavonoids Content
THIIH	Transcription factor II Human
TLRs	Toll-Like Receptors

TNF-α	Tumour Necrosis Factor-alpha
TPC	Total Polyphenols Content
UV-DAD	UV-Diode-Array Detection
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

1.1 *Cystoseira* genus

In Latin, the word *Cystoseira* means a chain of vesicles, describing the air bladders in the typical shape of the thallus. The genus is one of the oldest known brown seaweeds from fossils that were found in California and with an estimated age of 65.5 million years (Gardner, 1923). The earliest documented recognition of *Cystoseira* goes back to 1820 when Agardh classified the genus under Fucales order and sargassaceae family. The type designated in Bachelot de la Pylaie (1830) and the most recent taxonomic treatment adopted by Silberfeld *et al*. (2014) and Orellana *et al.* (2019) and the nomenclature followed that adopted by Guiry & Guiry (2020). The latest classification of the genus *Cystoseira* C. Agardh, 1820 (Fucales, Phaeophyceae) indicate that it includes approximately 165 species at present. Approximately 80% of the species occur along the Mediterranean and the Atlantic coasts (Guiry & Guiry, 2020). The standard method of determining relationships within the *Cystoseira* genus is based mainly on morphological characteristics (Jégou *et al.,* 2010). However, molecular tools have also been shown to be helpful in a taxonomical context, such as species phylogenetic relationships establishment (De Sousa *et al.,* 2019), mitochondrial, chloroplastic, and nuclear markers, ribosomal DNA analysis and Internal Transcribed Spacer (ITS2) (Rožić *et al.,* 2012; Draisma *et al.,* 2010; Jégou *et al.,* 2010).

Cystoseira species are hard to distinguish due to the complexity of their morphology, which environmental variables can influence. In general, *Cystoseira* thallus contains perennial basal parts, which are the holdfast and the main axis that are mostly flattened into 'foliar expansions' or basal leaves, which may be spinose or foliose (figure 1.1). The oldest thallus has an elongated primary axis called stipe, and over time, the primary laterals become relatively elongated (Mannino & Mancuso, 2009). The annual apical parts of the thallus may contain air-vesicles and represent the fertile parts and are called receptacles located at the tips of the branches above the aerocysts. The number of secondary axes increases exponentially with the length of the main axis, suggesting the existence of environmental constraints, which may impose severe restrictions on the unlimited growth of the central axis with the highest frequency within a maximum size of 44 cm (Garreta *et al.,* 2001). These algae contain two primary pigments in which are xanthophyll and fucoxanthin in addition to chlorophyll pigments. Xanthophyll and fucoxanthin pigments are responsible for the brown colour and are therefore called brown seaweeds. The pigments brown colour is essential for these algae

because it helps them adapt to the low light in the deep-sea and ocean (Ramus *et al.,* 1976; Prasanna *et al.,* 2010; Hurd *et al.,* 2014).

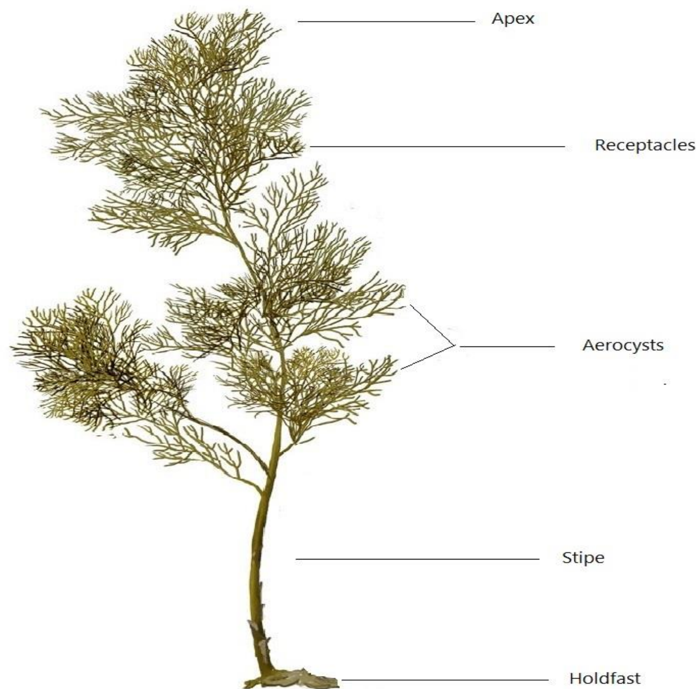


Figure 1.1 Morphological features of the *Cystoseira* genus.

Cystoseira species may differ in terms of the presence and absence of important features. For instance, *Cystoseira* colour varies among species and changes to some extent during the lifetime of the thallus. Some species have a dark brown colour, such as *C. barbata*, *C. barbatula*, *C. compressa*, *C. crinita*, *C. foeniculacea*, *C. crinitophylla*. Other species take the greenish-blue brown colour, such as *C. tamariscifolia* and *C. algeriensis* (Bermejo *et al.,* 2018; Bouafif, 2014). The attachment may be present or absent in *Cystoseira* species; for example, *C. barbatula*, *C. compressa*, *C. crinita*, *C. crinitophylla*, *C. foeniculacea* and *C. tamariscifolia* grow attached to the substrate, while other species such as *C. barbata* and *C. schiffneri* have free-living forms of thallus (Bouafif, 2016; González- Sampedro, 2017). Holdfast and axis with cylindrical primary branches are present in most *Cystoseira* species; however, they are absent in species such as *C. barbata* and *C. foeniculacea* (Gerloff & Nizamuddin, 1976)

Cystoseira identification is based on the way of the attachment to the substratum, the number and the shape of axes, the aspect of apices and tophules if present, the phyllotaxy and the morphology of branches, the occurrence and the arrangement of cryptostomata and aerocysts,

and the location and the morphology of reproductive structures (Bouafif *et al.,* 2016). The genus also is characterised depending on geographical distribution and ecological conditions. However, definitive taxonomic identification can be difficult because of the widespread cryptic inter-and intraspecific morphological variability within the genus (Garreta *et al.,* 2001). This notable morphological variability in *Cystoseira* has created many taxonomic changes in its member species, making it challenging to identify the exact limits separating species. As a result, the recognition of different species has included combined studies of reproductive parts morphology and phylogenetic analyses and chemistry (Montesanto & Panayotidis, 2001).

1.1.1 *Cystoseira* genus distribution

Cystoseira genus is distributed widely in the temperate regions dominating the benthic vegetation on unpolluted hard substratum, especially in the upper infralittoral zone. The genus plays an essential role in Mediterranean rocky coasts by raising the level of biodiversity, creating a shadow, providing nutrition, and offering habitat and interaction zones to other marine species. Multiple morphologically distinct variants of *Cystoseira* spp. have been previously recognised in the coastal regions of the Mediterranean Sea, Atlantic Ocean, Black Sea, Red Sea and Parisian Sea (Tsiamis *et al.,* 2016) (figure 1.2). The Mediterranean Sea is considered the hotspot for *Cystoseira* species, where they reach the highest diversity. Indeed, among the 165 species described worldwide, 74 % are present and endemic in the Mediterranean Sea, where they probably diverged after the Messinian salinity crisis about 6 million years ago and with an Atlantic ancestor (Cormaci *et al.,* 2012; Guiry & Guiry, 2020). The distribution of *Cystoseira* species can be affected by several factors such as coast depth, seawater nitrate and nitrite concentration, substrate, and seagrasses presence (Sales & Ballesteros, 2009).

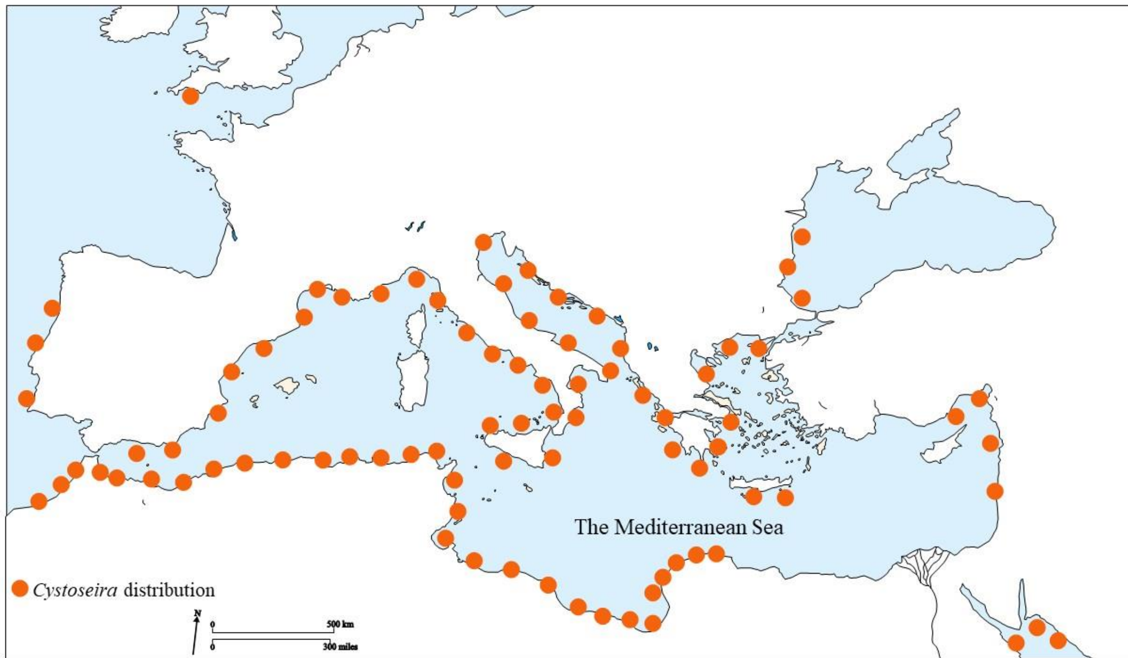


Figure 1.2 The map of *Cystoseira* distribution

Cystoseira tamariscifolia (Hudson) Papenfuss, 1950, exists solely in the Atlantic Ocean and the Mediterranean Sea entrance. This species shows the most development in the summer and spring season and occurs exclusively in the low nutrients and high oxygen waters in the intertidal and shallow subtidal zones (Garreta *et al.,* 2001; Celis-Plá *et al.,* 2014; Buonomo *et al.,* 2018). *Cystoseira crinita* (Duby) 1830 is a common species in most Mediterranean countries coasts that dominate the marine community in the upper shallow rocky areas 0.5 m depth with small waves and a high amount of sunlight (Ribera *et al.,* 1992; Garreta *et al.,* 2001; Blanfuné *et al.,*2016). It is worth mentioning that recently and while writing this thesis, there was a suggestion to rename and reclassify both *C. tamariscifolia* and *C. crinita* under a genus called *Carpodesmia* Orellana & Sansón 2019 (Orellana *et al*., 2019). Similar to C. *crinita*, *Cystoseira compressa* (Esper) Gerloff & Nizamuddin 1975 is common Mediterranean seaweed usually found in the upper regions of the intertidal zone on rocky and sandy substrates. It is a brown bushy alga, with an unbranched compact holdfast that develops to several short stipes end with flattened and cylindrical branches with flotation vesicles within the side (Schneider & Lane, 2007: Mancuso *et al*., 2016). All *C. tamariscifolia, C. crinita* and *C. compressa* (Figure 1.3) have been reported in many studies to contain a high content of bioactive metabolites such as polyphenols, flavonoids, polysaccharides, lipids and peptides (Custódio *et al.,* 2016; Mhadhebi *et al.,* 2011; Mandal *et al*., 2007; Bennamara *et al*., 1999). These bioactive

compounds represent a huge opportunity for researchers to discover novel natural alternative drugs for many diseases, including cancer and immune conditions.

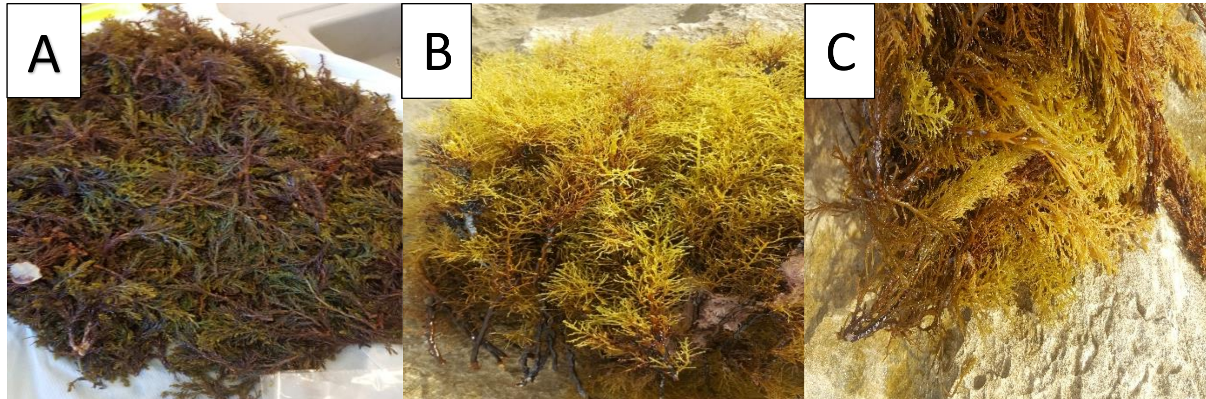


Figure 1.3 Pictures of *Cystoseira* species; (A) *Cystoseira tamariscifolia* (Hudson) Papenfuss1950, (B) *Cystoseira crinita* Duby 1830 and (C) *Cystoseira compressa* (Esper) Gerloff and Nizamuddin 1975

1.1.2 Cultivation and harvesting

Several protocols can be applied for *Cystoseira* culturing, including transplanting, tethering to other sea plants, germlings, entangled in nets screwed into the rock, attached to plastic meshes, fixed to ceramic plates, both detaching pieces of rocks bearing adult individuals and directly in holes drilled into the rocks (Perkol-Finkel *et al.,* 2006; Piazzi & Ceccherelli, 2019). *Cystoseira* generates a high number of gametes and zygotes under optimal conditions; therefore, the starting cultivation of germlings from fertile receptacles is a useful strategy for *Cystoseira* farming without depleting natural populations (Falace *et al.,* 2018; De La Fuente *et al.,* 2019). *Cystoseira* zygotes and germlings settle under adult plants, protected from high irradiance and other stressors. In nature, such community self-protection could be particularly crucial during spring-summer (Falace *et al.,* 2018). Therefore, capitalising on the reproductive season of the target species of *Cystoseira* could help optimising reforestation efforts. Another attempt for *Cystoseira* cultivation was performed by culturing *C. barbata* parts in the field using a variety of plastic plates that were located in areas with high settlement potential, but post-settlement survival probability was low (Perkol-Finkel *et al.,* 2006). Non-destructive restoration techniques, which avoid the depletion of threatened species in donor populations, were conducted in *C. amentacea* and *C. stricta* tiles in the north-western Mediterranean. In a month,

over 40% of the rocky area was covered with *Cystoseira* juveniles, which reached approximately eight mm in total length and survived the storms and micro-grazing (Vandendriessche *et al*., 2007; De La Fuente *et al.,* 2019). Marine Protected Areas give better protection to healthy forests than non-managed sites. They could be a source of propagules for natural recovery or for the non-destructive artificial restoration of nearby damaged forests. Cultivation involving a reproductive cycle, with alternation of generations, is necessary for many seaweeds; for these, new plants cannot be grown by taking cuttings from mature ones (Bak*,* 2019).

Other important drivers of success would include raising public and political awareness, legal actions and enforcing MPA management plans (Gianni *et al.,* 2016). In the case of *Cystoseira* species, although the vegetative part is developed annually, the permanent part usually has a slow growth rate, from 0.5 cm/year in *C. zosteroides* to 2 cm in 9 months in *C. crinita* (Ballesteros *et al.,* 2009; Sales & Ballesteros, 2012). The development of the vegetative part in deep-water species such as *C. zosteroides* begins earlier than for shallow-water species such as *C. crinita, C. barbata* and *C. mediterranea*. Researchers also suggest that due to the complementarities of habitats and synergies between habitats, mixed habitats may be more productive than a single habitat. (Ballesteros *et al*., 2009; Cheminée *et al.,* 2013; Ounanian *et al.,* 2017).

1.1.3 Seasonal and locational variation in bioactive compositions of *Cystoseira*

Cystoseira in temperate regions is exposed to the high stress of seasonal environmental changes that suggest *Cystoseira* might have developed more efficient photoprotective mechanisms to tolerate environmental stress than species from other biogeographical regions. In response to seasonal variations of both abiotic and biotic conditions, *Cystoseira* may produce and accumulate large quantities of metabolites that function for adaptive growth and defence mechanisms. At the early stages of the long-life cycle of *Cystoseira* alga, the flat leafy basal parts of *Cystoseira* develop into radially scattered apical branches in which intercalary air vesicles are usually located in the terminal forked receptacles. In the apex, most cryptostomata and conceptacles are bi-sexual, with the oogonia interior and the antheridia usually in a ring below the ostiole (González-Sampedro, 2017). *Cystoseira* species have a monogenetic diplontic life cycle, so the unique haploid stage is found in the gametes, which merges forming a heavy zygote (Figure 1.4). They are characterised by high reproductive potential, with abundant, large, heavy and quickly sinking eggs and zygotes. This reproductive strategy favours the formation of dense mono-specific assemblages but limits the dispersal ability of the species (Ounanian *et al.,* 2017).

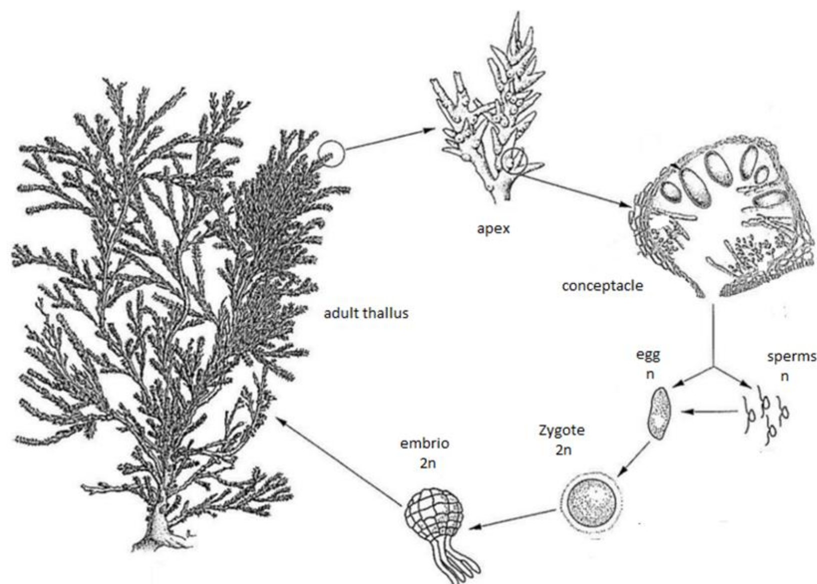


Figure 1.4 The life cycle of *Cystoseira* (from Garreta *et al*., 2001, and Grech, 2017, modified)

Cystoseira thallus is perennial and plurennial but with seasonal changes in shape and biomass during the year, although fertile receptacles are generally present in spring-early summer. Microscopic zygotes and juveniles, under optimal conditions of light, hydrodynamic and trophic conditions, take up to several months to reach the macroscopic size, and the growth may be delayed under less favourable conditions (Gunnill, 1980; Schiel & Foster, 2006). The potential for delayed growth in early life stages has important implications for population replenishment. The reproductive season can significantly vary between species and regions (Schiel & Foster, 2006). For those regions where there is a marked seasonality, like in the Mediterranean, reproduction begins at the productive season, usually spring, when nutrients are more abundant and there is enough light. Shallow species such as *C. barbata* and *C. mediterranea* usually remain fertile until late summer, while *C. zosteroides* is only reproductive from March to early June, and *C. crinita* is fertile from autumn to early spring in the Balearic Islands (González-Sampedro, 2017). Many natural factors can influence the survival of early stages, such as light and temperature or sediment load as well as human-derived impacts. In the case of some long-lived species such as *C. zosteroides*, recruitment can be very scarce over long periods (Ballesteros *et al.,* 2009).

The adult *Cystoseira* thallus has a high degree of seasonality in total biomass which generally shows to be higher in summer than winter in most areas. However, in some areas, such as the western Mediterranean, *Cystoseira* species show less seasonal variation compared with other regions. (Kaehler & Kennish, 1996; Mannino *et al.,* 2017). These variations are linked to variable abiotic and biotic parameters such as Light, UV-radiation, temperature, nutrients, depth, salinity, global warming and pollution (Schmitt *et al.,* 1995; Martí *et al.,* 2004), predation, fouling, growth stage and trophic competition (Ballesteros *et al.,* 2009; Marinho-Soriano *et al.,* 2006). Indeed, the effects of those parameters are also obvious on the biosynthesis of both primary and secondary bioactive metabolites, but the trends cannot be clearly elucidated because of the lack of a full set of controlled variables in nature (Airoldi, 2003). In most cases, the combination of several environmental conditions is more likely to have a major impact on the seasonal variation on both primary and secondary metabolites; for example, in the summer, light and temperature are inherently correlated (Zatelli *et al.,* 2018). In contrast with the open oceans, the Mediterranean is relatively high salinity, temperate to warm sea, extreme limited tidal rate, and oligotrophic waters. These conditions could limit the growth of kelps in the Mediterranean; however, these environmental factors seem to be suitable for the genus of *Cystoseira,* which explains why it is widespread in the Mediterranean (Sales

& Ballesteros, 2009). Photoprotectors produced in *Cystoseira* tissues help this genus to spread widely in temperate regions. Polyphenols in temperate regions are used as photoprotector agents against the large daily and seasonally variation of solar radiation, especially UV light. They also contribute to protecting seaweeds from the toxic effect of heavy metals, grazing and bacterial infections (Stengel *et al.,* 2011). Polyphenol levels are associated with the stress of environmental conditions, although further variations can also occur with life stages and within individuals. In particular, *C. amentacea* has been reported to have higher values of total polyphenols content in the winter and the lowest in the summer (Mannino *et al*., 2016). However, in both periods of the year, phenolic content in *C. amentacea* showed higher values when involved in protection mechanisms against grazers, epiphytes and UV radiation. (Mannino *et al.,* 2016; Mannino *et al.,* 2014; Mannino *et al.,* 2017). *C. foeniculacea* collected from the Turkey coast showed the highest eckol content in summer, while abscisic acid concentration was higher in spring. The study suggests that the accumulation of the bioactive compounds could be related to tolerance to increased seawater temperature and epiphytic flora variations during summer (Kozak *et al.,* 2020). Another study by Fariman *et al.* (2016) showed that *C. indica* collected from the Iran coast produced a significant amount of fucoxanthin in summer, which is possibly due to oxidative and thermal stress, while total lipid in Autumn was higher compared to the rest of the year.

The seawater temperature and the exposure duration to the sunlight play an important role in the chemical composition variation in brown seaweeds in different locations. For instance, an obvious spatial variation in bioactive compounds was found in *C. barbata* and *Cystoseira crinita* from the Black Sea when compared with the same species in the Mediterranean. This variation was also linked to human activities and the chemical characteristic of the coastal zone rocks. (Kravtsova *et al.,* 2015). Also, *Cystoseira* total annual production of branches and cauloids was higher than species living in conservation areas but much lower than that of *Cystoseira* species growing on exposed shores (Munda *et al.,* 1990). Generally, local and regional factors interact with physical variables and biotic environments to determine the patterns of spatial distribution and the biochemical components of seaweed species (Vergés *et al.,* 2009).

1.1.4 *Cystoseira* bioactive composition

Cystoseira is one of the largest suppliers of unique bioactive primary and secondary metabolites, but its possibility of drug development has stayed relatively unexplored. *Cystoseira* has a traditionally medical value in many countries and has been in the market for decades as a traditional diuretic drug in Oman and Iran (Gazor *et al.,* 2016). Many academic researchers and drug institutions have been developing drugs from metabolites isolated from the *Cystoseira* genus. Some of which are already in the market, and others reached the second phase of the clinical trials (Begum *et al.,* 2018). *Cystoseira* primary compounds, namely carbohydrates, proteins, lipids and nucleic acids, are the essential products that result from the photosynthesis process and are essential for whole thallus growth and reproduction. Several studies have isolated primary bioactive compounds from the *Cystoseira* genus (Mandal *et al.,* 2007; Bennamara *et al.,* 1999; Vizetto-Duarte *et al.,* 2016; Ayyad *et al.,* 2003; Hamdy *et al.,* 2009; Ozdemir *et al.,* 2006; Sellimi *et al.,* 2017; De Sousa *et al.,* 2017; Celis-Plá *et al.,* 2017). For instance, *C. tamariscifolia* in Portugal presented total carbohydrates at a concentration of 54% from the dry weight and high protein content, with around 13% of DW. *C. nodicaulis* and *C. compressa* had total carbohydrate levels of 73% of DW and protein contents of 9–10%. The total lipids concentration was 11% of DW in *C. baccata*, followed by *C. tamariscifolia* 10% of DW. The value of total protein obtained was 13% of DW, and all *Cystoseira* species had polyunsaturated fatty acids at concentrations ranging between 29% and 46% of DW from total fatty acids measured. (Vizetto-Duarte *et al.,* 2016a). A high level of sugars and fats content was seen in *C. barbata* samples from Bulgarian coasts, at concentrations 25.6 g kg^{-1} and 9.6 g $kg^{-1,}$ respectively (Manev *et al.,* 2013). One of the most bioactive polysaccharides is fucoidan which was detected in many *Cystoseira* species at high concentrations. For instance, an average of 16% of the total polysaccharides yield in *C. crinita*, *C. compressa*, and *C. sedoides* was found to be fucoidan (Ammar *et al.,* 2015). Secondary metabolites are not essential for organism growth but are produced to confer a selective advantage to the organism (Andrade *et al.,* 2013; Lopes *et al.,* 2012). The extreme conditions of the marine habitats, such as salinity fluctuations, temperature changes, competition on nutrients, solar irradiation, and water pressure, could lead to the increase of the secondary metabolites production through the development of chemical defence mechanisms. This has led to substantial variability of substances missed in terrestrial plants (Spavieri *et al.,* 2010; Vizetto-Duarte *et al.,* 2016b; Patel, 2018). Secondary metabolites include terpenoids, meroterpenoids, steroids, phenolic compounds and pigments correlated with a therapeutic effect have been isolated from many

Cystoseira genus (De Sousa *et al.,* 2017; Custódio *et al.,* 2016; Lopes *et al.,* 2013; Zbakh, 2019; Salimi *et al.,* 2018). For instance, *C. crinita* from the Black Sea were shown to have a concentration of 58.5% and 25.4% of fucosterol and 24-ethyl-cholest-5-en-3β-oL, respectively (Kamenarska *et al.,* 2002). Bulgarian *Cystoseira barbata* had a large polyphenol content with 66.9 µg mg^{-1} dry weight (Manev *et al.,* 2013). Total phenol and flavonoid contents in *C. barbata* and *C. compressa* extracts collected from the Adriatic Sea were 39.96-81.28 µg mg^{-1} and 20.85-64.58 µg $mg^{-1,}$ respectively (Kosanić *et al.,* 2015). A variety of both primary and secondary bioactive metabolites have been isolated from a wide range of *Cystoseira* species in many studies, which are summarised in table (1.1).

Table 1.1 *Cystoseira* biochemical compounds exhibiting biological activity.

Cystoseira species	Compound class	Compound name	Activity	Reference
C. tamariscifolia	Terpenoids	Isololiolide	Antiproliferative, cytotoxic, apoptosis	Vizetto-Duarte *et al.,* 2016
	Meroterpenoids	Demethoxy cystoketal chromane, Methoxybifurcarenone, tetraprenyltoluquinols	Antiproliferative, Antifungal, anti-bacterial, protecting a human dopaminergic cell line cytotoxicity	Bennamara *et al.,* 1999, Vizetto-Duarte *et al.,* 2016, Custódio *et al.,* 2016
	Steroids	Fucosterol	Antioxidant, cholinesterase inhibitor, anti-diabetic, antifungal	Andrade *et al.,* 2013, Pérez *et al.,* 2016
	Phenolic Compounds	Fucodiphloroethol, Phloroglucinol	Antioxidant, HAase inhibitor	Andrade, *et al* 2013, Stiger-Pouvreau *et al.,* 2014, Ferreres *et al.,* 2012
	Carbohydrates	Mannitol	Antioxidant, anti-diabetic, cholinesterase inhibitor	Andrade *et al.,* 2013
	Fatty Acids	Myristic acid, Margaric acid, Pentadecyclic acid, Lignoceric acid, Palmitic acid, Palmitoleic acid, Oleic acid	Antioxidant, Anti-inflammatory, cancer preventive, androgen inhibition, anti-diabetic, cholinesterase inhibitor, antifungal, anti-bacterial	Andrade *et al.,* 2013, Vizetto-Duarte *et al.,* 2015
	Pigments	Proline,	Anti-diabetic, cholinesterase inhibitor,	Andrade *et al.,* 2013
C. crinita	Terpenoids	3,7-Dimethyl-1,6-octadiene-3-ol-2-aminobenzoate, Hexahydrofarnesylacetone, Dihydro-3-hydroxy-3-hydroxymethyl-2(3H)-furanone, Dihydroactinidiolide	Cytotoxicity	Fisch *et al.,* 2003
	Meroterpenoids	methyl hydroquinone, 5-Oxo-cystofuranoquinone, Tetraprenyltoluquinols, tetraprenyltoluquinones	Antioxidant, cytotoxic,	Fisch*, et al.,* 2003.
	Steroids	Stigmasterol, Isofucosterol, Saringasterol Fucosterol, Brassicasterol	Antioxidant, anticancer, antimicrobial	Milkova *et al.,* 1997, Pérez *et al.,* 2016.

	polyphenoles	Benzoic acid, 2,4-Bis-(1,1-dimethylethyl) phenol	Antioxidant, anti-inflammatory	Fisch *et al.,* 2003.
	Carbohydrates	Fucoidans, Uronic acid	anti-allergic, anticancer, Anti-herpetic	Mhadhebi *et al.,* 2014
	Fatty Acid	a-Linolenic acid, c-Linolenic acid	Anti-inflammatory, endocrine precursor, skin protective, anti-rheumatoid arthritis, anti-multiple sclerosis, schizophrenia, preventive, premenstrual, syndrome preventive, infant diet supplement	Vizetto-Duarte *et al.,* 2015
C. compressa	polyphenol	Phlorotannins	Antioxidant, anti-inflammatory, antiproliferative	Kosanić *et al.*, 2015; Güner *et al.*, 2015,
C. sedoides	polyphenol	Phlorotannins	Antioxidant, anti-inflammatory, antiproliferative	Mhadhebi *et al.,* 2014, Abdelhamid *et al.,* 2019
C. myrica	Carbohydrates	sulfated polysaccharides, Fucoidans. Mannitol.	Anti-inflammatory, oedema inhibition, gastroprotective, antioxidant, anti-allergic, anticancer, Anti-herpetic	Mhadhebi *et al.,* 2014
	Terpenoids	Cystoseirol monoacetate, Dictyol F monoacetate, Dictyone, Isodictytriol,monoacetatePachydictyol	cytotoxic	Ayyad *et al.,* 2003
	Steroids	Cholest-4-ene-3,6-di-one, 3-Keto-22-epi-28-nor-cathasterone	Cytotoxic	Hamdy, *et al.,* 2009
C. barbata	Steroids	Fucosterol, Saringasterol	Antioxidant, cholinesterase inhibitor, anti-diabetic, anticancer, anti-microbial.	Ozdemir, *et al.,* 2006, Hannan, *et al* 2020
	Phenolic Compounds	Phenol-2,4 bis-(1,1-dimethylethyl),	, anti-intercellular bacterial, anti-biofilm,Antioxidant,	Ozdemir, *et al.,* 2006
	Fatty Acids	Caproic acid, Lauric acid, Palmitic acid, Myristic acid, heptadecenoic, erucic, and docosahexaenoic, acids, stearic acid, Heneicosylic acid, Tricosylic acid, Oleic acid, alpha-Linolenic acid	Anti-inflammatory, cancer preventive, anti-androgenic, anti-diabetic, cholinesterase inhibitor, antifungal, anti-bacterial, antioxidant Anti-inflammatory, anti-bacterial	Panayotova & Stancheva, 2013, Vizetto-Duarte *et al.,* 2015, Lopes *et al.,* 2013 and Brown *et al.,* 2012
	Pigments	Astaxanthin, a-Tocopherol, Retinol, Ergocalciferol, fucoxanthin	Antioxidant, antitumoral, cellular signalling, gene expression regulator, antigenotoxic, Anti-aging, Cytotoxic, endocrine regulator, Ca/P homeostatic.	Bai *et al.,* 2005, Sellimi *et al.,* 2017, Panayotova & Stancheva, 2013

C. usneoides	Meroterpenoids	Cystodione, Amentadione-10-methyl ether, Usneoidone Z	Anti-inflammatory, antioxidant,anticancer	De Los Reyes *et al.,* 2013, De Los Reyes *et al.,* 2016, Zbakh, 2019.
	Phenolic Compounds	Fucotriphloroethol	Antioxidant, HAase inhibitor	Ferreres *et al.,* 2012
	Carbohydrates	Mannitol	Antioxidant, anti-diabetic, cholinesterase inhibitor	Andrade *et al.,* 2013
	Amino acids and protein	Proline	Anti-diabetic, cholinesterase inhibitor	Andrade *et al.,* 2013
C. nodicaulis	Carbohydrates	Mannitol	Antioxidant, anti-diabetic	Andrade *et al.,* 2013
C. abies-marina	Meroterpenoids	Cystoazorol, Cystoazorone, Cystomexicone	Antioxidant, cytotoxic	Gouveia *et al.,* 2013
	Phenolic Compounds	Benzoic acid	Antioxidant, anti-inflammatory, antifungal	Gouveia *et al.,* 2013
C. humilis	Phenolics	Phloroethol, Phloroglucinol	Antioxidant,	Stiger-Pouvreau *et al.,* 2014
C. baccata	Meroterpenoids	Tetraprenyltoluquinols	Antileishmanial	De Sousa *et al.,* 2017.
	Phenolics	phlorotannins	Anticancer	Duarte, 2016

1.2 Cancer diseases

Cancer is a group of illnesses involving uncontrolled cell growth with the possibility of killing normal cells and spreading rapidly within the body. According to the International Agency for Research on Cancer (IARC), 2018, there were approximately 18.1 million new cancer cases, with 9.6 million deaths globally, and the global burden is estimated to rise to 27.5 million new cancer cases by 2040. Prostate, lung, pharynx, oesophagus, tongue and stomach cancers were the most common cancers found in males, while cervical, breast, ovarian, oesophageal and oral cancers were common among females (Hussain *et al.,* 2016; Bray *et al*., 2018). In most cases, cancer causes are not clear; however, cigarette smoking, heredity, lifestyle, viruses, exposure to carcinogenic chemicals and ultraviolet light, hormones, and immunology play directly or indirectly an important role in cancer (Zaridze, 2002). However, experts generally agree that preventive measures are the best way to follow to reduce deaths. This could be by stopping smoking, avoiding exposure to the UV rays of sunlight, and following a healthy diet that is low in fat, high in natural fibre, and rich in fruits and vegetables could significantly reduce the risk of conducting cancer (Martin-Moreno *et al*., 2008). Moreover, the good management of chemotherapeutic medications that include dietary supplements such as hormones and vitamins along with synthetic drugs such as aspirin and other agents used daily for therapeutic purposes could also increase the chance of survival from cancers (Adami *et al.,* 2001).

Prostate cancer is one of the most common cancer-associated deaths in men. It is the second cause of death in men around the world, with roughly 300 thousand deaths per year (Thakur *et al.,* 2018). Symptoms of prostate cancer depend on the stage at which cancer is in; the early stages symptoms may not be visible, and here is challenging to diagnose. As the disease develops, the patient has problems with urination due to the pressure of a cancerous tumour on the bladder or urethra (Nitti *et al.,* 1997; Danielson *et al.,* 2010). It may include urinating difficulty, urinating intermittently, or feeling that the bladder is not fully discharged. Furthermore, there can be chronic pelvic pain and difficulties in urination and loss of control of urination, poor sexual performance or complete impotence, and the occurrence of fractures in some bones (Yadav *et al.,* 2018; Drudge-Coates *et al.,* 2018). These symptoms may be correlated with other conditions in the prostate gland, such as acute or chronic inflammation, benign prostatic hyperplasia or other urinary tract disorders. The complications can be resulting from the disease itself or as a result of the treatment. Psychological complications, such as depression, may impact the patient (Taneja *et al.,* 2017; Pirl & Mello, 2002). Prostate cancer can be accompanied by other signs, such as blood in the urine, blood in the semen, discomfort

in the pelvis, swelling of the legs, and bone and spine pain, which are advanced stages of the disease (Lemanska *et al.,* 2018). The exact cause of the prostate tumour is not yet known, and why its types behave differently. The researchers believe that a combination of several different factors is responsible for the development of the disease, including age, genetics, race, hormones and nutrition (Nascimento-Gonçalves *et al.,* 2018).

Leukaemia is abnormal leukocytes that prevent normal white blood cells activity and proliferation, and it is classified into several types, such as acute leukaemia, chronic leukaemia, and acute, chronic lymphoblastic leukaemia. Acute leukaemia is common cancer in childhood and leads to a tremendous loss of life. It works very quickly in the body and may kill a human being at any moment (Nordqvist, 2017). A large number of infected white blood cells prevent stem cells from doing their work in the blood. It also increases the incidence of anaemia and the inability to eliminate diseases. Chronic leukaemia is the most common type of leukaemia in developed countries and affects senior men more than women. It tends to progress slowly over the years and can be classified into chronic myeloid leukaemia and chronic lymphoblastic leukaemia (Vardiman *et al.,* 2009; Getta *et al.,* 2015; Mwirigi *et al.,* 2017). There are no specific causes of leukaemia, but several habits are likely to make a person prone to leukaemia. The daily exposure of chemicals, genetic factors, and smoking is a potent catalyst for the destruction of white blood cells (Wiemels, 2012; Ding & Bao, 2013). Leukaemia symptoms are often neck and armpit lymph glands inflammation, rapid and sudden loss of weight (Terao *et al.,* 2011). Leukaemia also increases the size of the liver, causes tiredness feeling and fatigue, red skin spots, and many bruises and internal bleeding (Karimi *et al.,* 2008). Mantle cell lymphoma is also blood cancer that develops when white blood cells called B-lymphocytes become abnormal and fail to fight infectious diseases and stop helping fluids move through the body (Hallek, 2019). The B-cell lymphomas usually accumulate in lymph nodes and cause swollen lymph glands, but they can also impact the bone marrow, bowel and spleen (Siegel *et al.,* 2020).

1.2.1 The need for natural products for new anticancer drugs

The therapeutic efficacy of existing antitumor medications has been impeded by their poor therapeutic value and lack of medical effectiveness in humans. Thus, employing natural products to improve anticancer therapy has been approached using the marine environment as a source. Traditionally, cancer treatment focussed on radiology, surgery, high-dose chemotherapy, hormonal therapy and immune therapy (Senthebane *et al.,* 2017). Reconstruction surgery is performed when the affected body part can be replaced, while hormonal therapy is sometimes used to prevent cancer cells from getting the hormones they need to grow after a surgery (Voda, 1994). Radiotherapy has been used to treat a number of cancer cases by employing ionising photon and particle beams to cure cancer, whether as primary curative or tumour-related signs therapy (Nair *et al.,* 2001; Stupp *et al.,* 2005). Chemotherapy is a systemic therapy that works through the entire body to eliminate cancer cells, unlike surgery and radiation therapy. It is usually used as a single or a combination of drugs to kill any invisible residual cancer cells in the body after the surgery (Rick *et al.,* 2019). However, major issues such as anti-drug reactions, poor effectiveness and toxic effects in humans as compared to behaviour in animal models, or variability in such problems across individuals within a group, sometimes arise in metabolic drug issues as well as the high cost of cancer treatments (Coffelt & de-Visser, 2015;). Thus, now the aim is to develop a natural product as a cancer drug that matches relatively low side effects with maximum anticancer activity from abundant sources. Early-stage of developing such a cancer treatment has resulted in several new cytotoxic agents (Hossain & Andreana, 2019; Moody *et al.,* 2020). Therefore enhanced *Cystoseira* bioactive compounds for anticancer therapy is could be hugely useful for the safe and affordable treatment of cancer patients.

1.2.2 Brown seaweed as a source of anticancer bioactive compounds

Recently, natural products from the marine environment and in particular brown seaweed, have been highlighted for their importance as an anticancer bioactive compounds source. Most of these bioactive compounds are generally safe and abundantly exist in brown seaweeds, making them a promising strategy of tumour therapy (Zubia *et al*., 2009; Gutiérrez-Rodríguez *et al*., 2017). Many compounds such as polysaccharides, polyphenols, flavonoids, terpenoids and steroids have been isolated from brown macroalgae currently receive the most attention for their potential anticancer properties (Mhadhebi *et al.,* 2014; Alghazeer *et al.,* 2016; Abdelhamid *et al.,* 2019; Zbakh, 2019). Polysaccharides, for example, have been investigated by both pharmaceutical companies and academic researchers for anticancer drug development or drug design. Polysaccharides isolated from *Fucus* spp showed an antitumor and anti-metastasis activity in lung tumours (Alekseyenko *et al.,* 2007). Moreover, the sulphated polysaccharide fucoidan isolated from *Fucus vesiculosus* has shown to induce apoptosis via the mitochondrial apoptosis pathway, activated extrinsically by death receptor 5 (DR5) in HT29 and HCT116 colorectal cancer cell lines (Bai *et al.,* 2020). Fucoidan also exhibits a significant anticancer effect against the hepatocarcinoma cell line (Huh7); the suggested mechanism is that fucoidan causes cell cycle arrest at the G0/G1 phase (Senthilkumar *et al.,* 2013). Polyphenols from brown macroalgae also have been shown to have anticancer activity; for example, phlorotannins extracted and purified from *Ecklonia cava*, *Eisenia arborea*, *Ecklonia stolinifera* and *Eisenia bicyclis* exhibited potentiality as antitumor agents against several types of cancer cell lines (Gutiérrez-Rodríguez *et al.,* 2017). Cinnamic acid is another phenolic compound that was purified from *Padina gymnospora* and displayed an antiproliferative activity against HeLa and A549 human cell lines (Sali *et al.,* 2016). The alkaloid podophyllotoxin shows activity against lymphomas and brain tumours (Da Rocha *et al.,* 2001). Caulerpin is another alkaloid isolated from *Sargassum platycarpum,* also has significant *in vitro* anticancer activity against liver human cancer cell line HepG2 (Abdelrheem *et al.,* 2020). The pigment Carotenoids from the brown alga *Undaria pinnatifida* have anti-proliferation of human prostate cancer (Kotake-Nara *et al.,* 2001). Moreover, the Carotenoids fucoxanthin Purified from *Laminaria japonica*, *Hizikia fusiforme* and *Undaria pinnatifida* extracts was found to reduce colorectal aberrant crypt foci formation in mice and to induce apoptosis in human colon cancer cell lines (HT116) and colorectal cancer cells (CaCo2) *in vitro* via the activation of the potent cyclin-dependent kinase inhibitor p21 or the classic Bcl-2-dependent caspase cascade (Mei *et al.,*2017; Peng *et al.,*2011)

Among the brown macroalgae, *the Cystoseira* genus has also shown to have active anti-tumour metabolites in which were subsequently isolated and identified. These metabolites have proved effective in the treatment of human tumours include leukaemia, lung, colon and prostate. Some of which has entered phase II clinical studies for cancer treatment. For instance, the hexane fraction of *C. crinita* and *C. stricta* showed an anti-proliferation effect against Caco2 cells and HCEC cells line (Alghazeer *et al.,* 2016), and hexane fraction from *C. myrica* also increased apoptosis in Caco-2 and T47D cell lines (Khanavi *et al.,* 2010). Polyphenols are highly concentrated in *Cystoseira* and are interestingly correlated to anticancer activity (Abdelhamid *et al*., 2019). The mechanism of the anticancer effect of polyphenols can be by inhibiting iron absorption or cytotoxic pro-oxidants influence and affect the mitotic division (Ozdemir *et al.,* 2006). Curcumin, for example, inhibited the formation of the mitotic spindle in breast cancer by preventing normal microtubules from forming. Polyphenols have also been known to participate in decreasing angiogenesis and cancer cells adhesion and invasion (Reitzer *et al.,* 2018). The anticancer effect of polyphenols and flavonoids has been attributed to the Ortho-dihydroxy groups on the aromatic rings and also to the four bonding electrons in the structure (Grigalius & Petrikaite, 2017; Koşar, 2017). In general, the cytotoxic effect of the polyphenols to eliminate cancer cells is mostly by inducing apoptosis or by the activation of cell signalling enzymes of protein kinase-c (Sithranga & Kathiresan, 2010). Polyphenols could induce apoptosis in cancer cells via multiple mechanisms such as the activation of cytochrome c and caspases, the arrest of the cell cycle, and the modulation of signalling pathways Nuclear Factors-Kappa B (NF-*κ*B), Janus kinase (JAK)/ Signal Transducer and Activator of Transcription 3(STAT3)) which lead to tumour progression inhibition (Wang *et al.,* 2020). Fucoidan and fucoxanthin isolated from *C. mediterranea* in Turkey showed apoptosis on human breast MCF-7 and human prostate cancer cells PC-3 (Taskin *et al.,* 2010). They are functioning by targeting the lysosomal membrane and also induce cell necrosis in several tumour cells (Zorofchian *et al.,* 2014). Laminarin isolated from various species of the *Cystoseira* genus (Sellimi *et al.,* 2018; Guarda, 2019) can inhibit the proliferation of human colon cancer LoVo through a mitochondrial pathway as well as induce apoptosis (Ji *et al.,* 2013). Also, it showed complete inhibition of melanoma, breast-carcinoma growth and lung tumour cells colonisation (Miao *et al.,* 1999). Meroterpenes isolated from *C. usneoides* were found to exhibit antitumoural activities against lymphoma P-388, leukaemia L-1210 and Carcinoma A-549 cell lines (Urones *et al.,* 1992). Meroterpenoids were also isolated from *C. usneoides* and tested against colorectal cancer HT-29. They inhibited the growth of HT-29 malignant cells by arresting in the G2/M phase and induced apoptosis as well as significantly

inhibited the migration of colon cancer cells (Zbakh *et al.,* 2020). Cell cycle arrest in response to DNA damage is initiated by phosphorylation of tumour protein p53, which binds to The Cdk-Activating Kinase (CAK) or Transcription factor II Human (THIIH). The effect is further enhanced by the p53 modulated activation of CDK-interacting protein p21, which is a potent inhibitor of G1 cyclin-dependent kinases via blocking the phosphorylation of the cyclin-dependent kinase CDK-2 and CDK-4 (Abbas & Dutta, 2009). When p53 is mutated in most cancer cells, DNA damage becomes irreversible and cannot be recovered, leading to activated apoptosis (Nowsheen & Yang, 2016). The meroterpenoids also reduced the phosphorylation levels of extracellular signal-regulated kinase and phosphorylation levels of protein kinase B in HT-29 cell lines (Zbakh *et al.,* 2020).

1.3 Inflammation

Inflammation is an adaptive protective reaction that is triggered by noxious stimuli and conditions, such as infection and tissue damage. It is a basic mechanism for consists of a cascade of cellular and microvascular reactions that serve in any organ, but it is most easily observable in the skin and underlying tissues (Fujiwara & Kobayashi, 2005). It is usually companied with five essential elements which are erythema, swelling, rise in temperature, pain, and dysfunctionality (Ackerknecht, 1953; Du *et al.*, 2015). The inflammatory response is a well-ordered series of events that starts with the production of chemokines and soluble mediators such as cytokines by resident cells such as macrophages, vascular endothelial cells, dendritic cells, and interstitial fibroblasts. This process modifies the profile of local adhesion molecules, establishing a chemotactic gradient that draws cells from the circulation which usually leads to the appearance of the clinical symptoms of inflammation (Laura *et al.,* 2010; Pober & Sessa, 2014). Macrophages play a major role in the production, mobilization, activation, and regulation of inflammatory and immune effector cells. Their transition from a resting to an activated macrophage is a critical phase in the functional development of inflammatory macrophages. Among macrophages, M1 macrophages express numerous pro-inflammatory mediators including tumour necrosis factor-alpha (TNF-α), interleukins, reactive nitrogen and oxygen intermediates, which have a strong microbicidal and tumoricidal activity (Murray & Wynn, 2011). Their activation signals include T lymphocyte-derived cytokines, granulocyte-monocyte colony stimulating factor (GM-CSF), and TNF-α, microbial products such as lipopolysaccharide (LPS) released by gram-negative bacteria and Lipoteichoic acid (LTA), immune complexes, chemical mediators and extracellular matrix proteins such as fibronectin and interferon-gamma (IFN-γ) (Mills *et al.,* 2000). M1 macrophage could choose surface activation markers which can be used to identify M1 macrophages including CD80, CD86, CD64, CD16, and CD32. For instance, CD80 is a T lymphocyte activating antigen also known as B7, B7.1, or BB1. It has a molar mass of 60 kD. It works in tandem with CD86 to activate T cells and is crucial in autoimmune monitoring, humoral immune response, and transplantation response. CD80 is a T lymphocyte activating antigen also known as B7, B7.1, or BB1 and It has a molar mass of 60 kD. It works in tandem with CD86 to activate T cells and is crucial in autoimmune monitoring, humoral immune response, and transplantation response (Petro *et al.*, 1995; Sudan *et al.*, 2015). CD64, also known as high-affinity immunoglobulin gamma Fc receptor I, is involved in both innate and adaptive immune responses. CD32, also known as low-affinity immunoglobulin gamma fc region receptor II-b,

is a receptor that is involved in the phagocytosis of immunological complexes and the control of B cells antibody production (Figure 1.5) (Wynn *et al*., 2013; Wang *et al*., 2014).

Many variables influence M1 macrophage phenotypic and functional polarisation, STATs, interfering regulatory factors (IRFs), NF-κB, activating proteins (AP1), peroxisome proliferators activating receptors (PPAR-gamma), and cAMP response element-binding protein are signal transducers and transcriptional activators that influence macrophage polarisation (CREB) (Saliba *et al*., 2014). They work together to control the phenotype of macrophages and polarisation. There are several signalling pathways that affect macrophage polarization. For instance, The Notch pathway regulates macrophage polarization by controlling gene expression, thereby modulating the immune response. Myelo-derived macrophages activate Notch1 and NF-κB in response to LPS and Toll-like receptors (TLRs) activation, resulting in polarised M1 macrophages. One biological mechanism underlying Notch1-dependent M1 polarisation is NICD1-mediated transactivation of the M1macrophages gene, which results in Notch1 activation. (Xu *et al*., 2015). Also, IRF5 is associated with M1 macrophage polarization and can be stimulated by inflammation in atherosclerosis, and nitrification of IRF5 protein leads to inhibition of IRF5-targeted M1 macrophage signalling gene activation (Weiss *et al*., 2013). Moreover, The JAK-STAT signalling pathway is also closely related to the phenotypic activity of macrophages where IFN works through this signalling pathway. IFN-γ can induce polarization of M1 macrophages (Seif *et al*., 2017). The involvement of IFN mediated signalling pathways in macrophage polarisation is unknown, however, it is known that it can improve anti-inflammatory actions under specific situations (Hall *et al*., 2013). Distinct AKt kinases influence macrophage polarisation in different ways. Among these, the phosphatidylinositol-3-kinase (PI3K) pathway which is essential for macrophage survival. PI3K may activate AKt1, and AKt1 ablation leads to the polarisation of M1- macrophages (Sharif *et al*., 2019; Linton *et al*., 2019). In addition to the signalling pathways mentioned above, mitochondrial biosynthesis is involved in macrophage polarisation (Wang *et al*., 2014). The phrase activated macrophage refers to a cell that has an enhanced ability to destroy microorganisms, tumour cells, and produce cytokines (Gordon, 1998; Mantovani *et al.,* 2002; Slauch, 2011). Cytokines are small proteins that work as inflammation mediators, which affect the development and the path of numerous immunologic responses. They are classified based on their structure as well as their receptors structural into several groups of cytokines, namely interleukins, interferons, tumour necrosis factors, chemokines, growth factors, and colony-stimulating factors (Autieri, 2012; Ziegler *et al.,* 2019). They can

also be categorised into pro-inflammatory and anti-inflammatory cytokines depending on their role during the initial appearance at the injury or infection sites. Pro-inflammatory cytokines such as Interleukins IL-1β, IL-6, IL-8, IL-12, IL-18, IFN-γ, TNF-α, those cytokines mobilise immune system cells to multiply and release more cytokines in order to create an inflammatory cascade. Anti-inflammatory cytokines, such as Interleukins IL-10, IL-33, IL-6, IL-13, IL-10, IL-11, IL-4, IL-19 and Transforming Growth Factor B (TGFβ), specialise in inhibiting the inflammatory response (Opal & DePalo, 2000; Minutti *et al.,* 2019). Cytokines have redundant activities, which means one cytokine can involve in different functions, and similar functions can be activated by different cytokines. For instance, TNF-α and IL-6 can perform both pro and anti-inflammatory roles in the immune response (Zhang & An, 2007). Pro-inflammatory cytokine's main function is to initiate an immune reaction to infectious extracellular pathogens, and in particular, TNF-α and IL-1 which are often secreted by M1 macrophages, have a significant role in the infection pathophysiology (Petricevich, 2006).

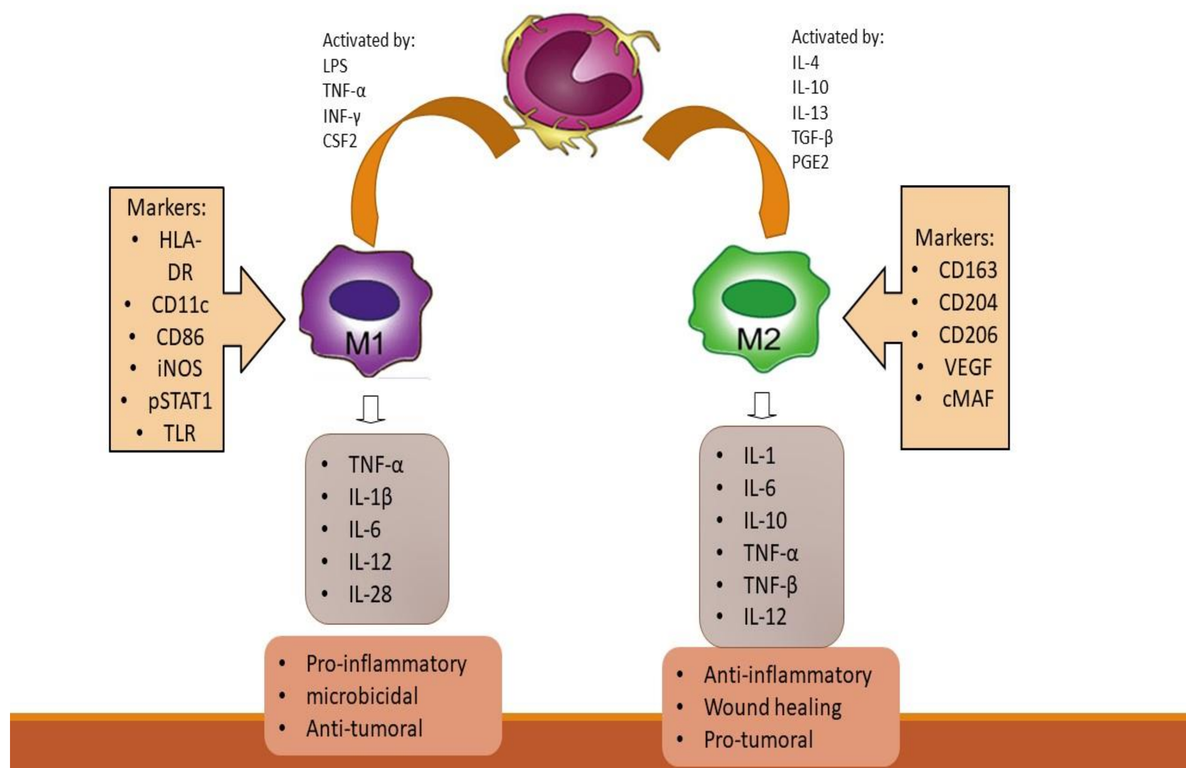


Figure 1.5 Macrophage activation during infection and inflammation.

A valid inflammation promotion required dissoluble cytokine bonds and particular membrane receptors like TLRs, which are single-pass membrane-spanning receptors usually expressed by macrophages and allow toxins to transmit their effects into the cell (Barton & Medzhitov, 2003). The cytokines and receptors set recognise exogenous and endogenous inducers include pathogen-associated molecular patterns (PAMPs), such as LPS and LTA (Moreau, 2016; Karbian *et al.,* 2020). It is worth noting that whereas PAMP variations are identified by the same pattern-recognition receptors (PRRs), they may activate separate signalling pathways. For instance, smooth or rough LPS variants activate either the MyD88-dependent or TRIF-dependent pathway. This suggests that the host distinguishes between various kinds of bacteria that contain LPS by activating separate signalling pathways (Kawasaki & Kawai, 2014). For instance, TLR4 functions as a receptor for LPS and forms a complex with Myeloid Differentiation Protein 2 (MD2) to interact with LPS. The creation of the TLR4-MD2-LPS complex activates the adaptor of myeloid differentiation factor 88 (MyD88) and initiates pro-inflammatory signalling pathways, including PI3K and protein kinase B activation (Akt or PKB). (Takeda & Akira, 2005; Fujiwara & Kobayashi, 2005). According to reports, Akt promotes inflammatory responses through the nuclear factor-B (NF-B) signalling pathway. Additionally, the mechanistic target of rapamycin (mTOR) pathway is required for signalling downstream of TLR4/Akt, where it plays a role in cell proliferation and immunological control. Both PI3K and mTOR inhibitors have been shown to decrease LPS-induced cytokine production in RAW264.7 cells by lowering Akt phosphorylation. Additionally, the mitogen-activated protein kinase (MAPK) pathway is critical for TLR4 signalling and consequent pro-inflammatory mediator synthesis such as TNF-α and IL-1β (Figure 1.6); (Autieri, 2012; Bergsbaken *et al*., 2009; Fang *et al*., 2017).

A successful inflammatory response includes the elimination of the infection cause and endotoxin removal, followed by inflammatory agents' resolution, and tissue repair is the ultimate goal of the immune system. It is vital that for balanced inflammatory, pro-inflammatory intercellular mediators must be replaced by resolving mediators such as pro-like lipoxins and lipids. Also, signalling pathways that activate white blood cells and the apoptosis process have to end (Buckley *et al.,* 2014). Moreover, most importantly, the polarisation of pro-inflammatory classically activated M1 macrophages into anti-inflammatory alternatively activated macrophages (M2 type) by IL-10, TGF-β and chemokines has to begin (Hirayama *et al.,* 2018; Atri *et al.,* 2018). In the final phase, macrophages remove pathogens, injured cells, and dying neutrophils by phagocytosis mechanism. Once macrophages complete their task at

the inflammation site, they are removed by the lymphatic system, whereas the fluid pressure on lymphatic capillaries increases, forcing capillaries to open their one-way valves. Secreted lymph contains macrophages passes through several lymph nodes and is filtered clean before it returns to the bloodstream (Hampton & Chtanova, 2019). Once the site is cleared, the immune cells stop producing pro-inflammatory chemicals, and as a replacement, they began generating anti-inflammatory mediators that successfully accelerate the end of inflammation (Freire & Van Dyke, 2013). This is the favourable outcome of inflammation, and on the contrary, failing to resolve inflammation leads to the development of chronic inflammation, which continuously deals damage to healthy tissues (Branco *et al.,* 2018). The consequences of the inflammatory responses malfunction to infection are microbial survive and tissue damage, and septic shock leads to severe respiratory distress syndrome or multi-organ failure. Nevertheless, in some cases, the immune system may become disturbed and begin to attack the body and its healthy cells by mistake (Bermejo-Martin *et al.,* 2014)

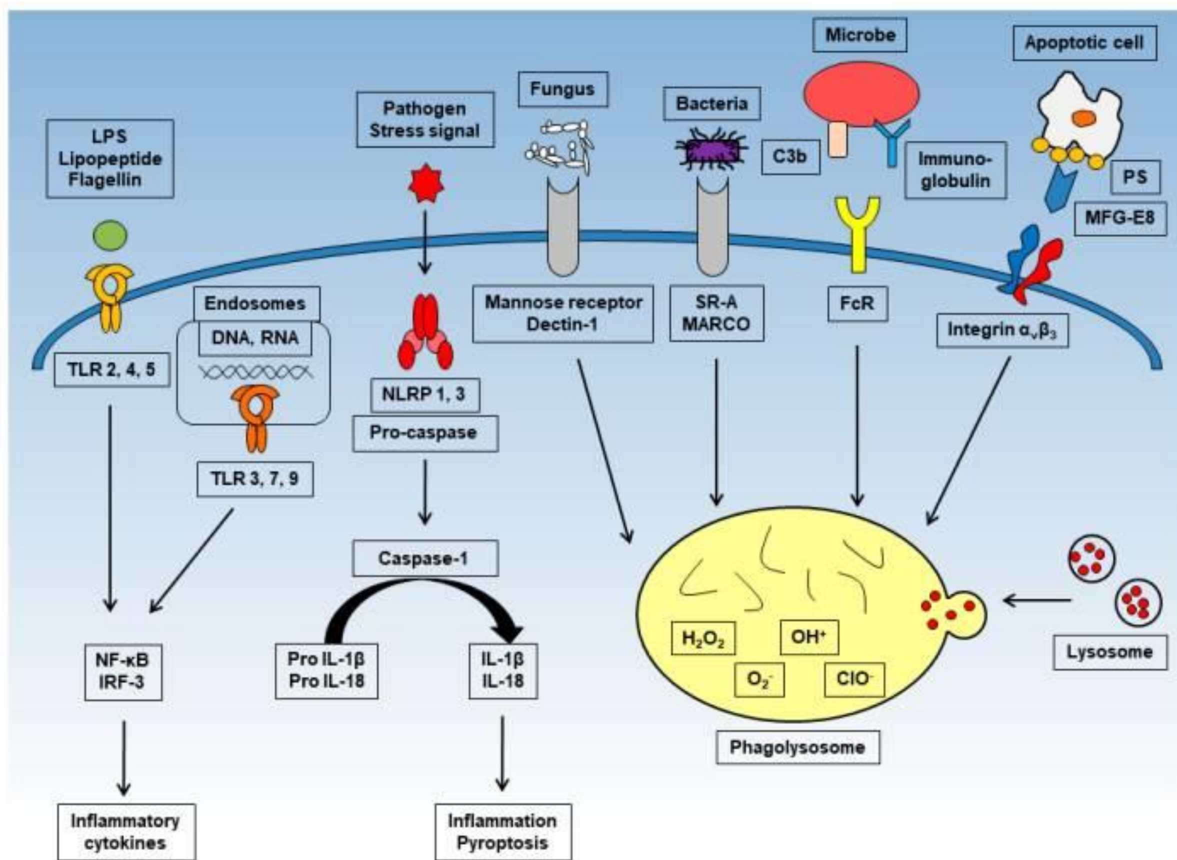


Figure 1.6 Cellular pro-inflammatory response pathways (Hirayama *et al.*, 2018)

1.3.1 Inflammatory disease and the need for therapy

Inflammatory disorders arise when inflammation becomes uncontrolled and disturb the homeostasis of one or more physiological systems that can give rise to the pathophysiological mechanisms underlying many acute and chronic inflammation diseases. There are more than 80 immune disorders that occur as a result of the immune system distributions, including cardiovascular diseases, diabetes, rheumatoid arthritis, lupus erythematosus, eczema, asthma and allergic rhinitis cancer, bowel disease and even depression (Davidson *et al*., 2001; Chen *et al.,*2017; Parikh *et al*., 2020; Henriksen *et al.,* 201; Nutten, 2015; Bantz *et al.,* 2014). Immune responses, both innate and adaptive, play a critical role in the genesis and progression of many cardiovascular disorders. The idea of atherosclerosis as a chronic inflammatory disease is widely regarded as the fundamental cause of coronary artery disease, stroke, and peripheral vascular disease. The pathogenesis of atrial fibrillation, the most prevalent heart arrhythmia, is also influenced by inflammation. Defects in inflammation resolution accelerate the development of atherosclerosis to susceptible plaque, and aberrant immune responses can contribute to cardiac remodelling following a myocardial infarction (Colin & Staels, 2015). The progression of vascular disorders is influenced by complex cellular and inflammatory interactions. When endothelial cells are exposed to cytokines, they undergo significant functional changes that include gene expression and de novo protein synthesis. Cytokines have an important role in the functional reprogramming of endothelial cells, especially in individuals with chronic vascular inflammation. TNF-α, IL-1, IL-6, and IFN- are stimulatory cytokines and growth factors produced by the intercellular network of dendritic cells, T-lymphocytes, macrophages, and smooth muscle cells, which stimulate the development of functional and structural vascular alterations. Proinflammatory cytokines enhance oxidative stress, inhibit Endothelial Nitric Oxide Synthase (eNOS) bioactivity, and cause endothelial cell death (Kofler *et al*., 2005). Patients with hypertension also showed a high level of IL-6 and TNF-α distribution and demonstrated an independent association between increased inflammatory markers, such as CRP, IL-6 and TNF-α, and hypertension (Solak, *et al*., 2016). The most frequent organ-specific autoimmune condition is Autoimmune Thyroid Disease (AITD). Thyroid autoantigens such as thyroid peroxidase (TPO), thyroglobulin (TG), and Thyroid Stimulating Hormone Receptor (TSHR) cause AITD to develop owing to a lack of immunological tolerance and reactivity of TSHR (Kohanim *et al.,* 2020). In Graves' disease, the immune system attacks the thyroid, leading to hyperactivity and release of more hormones, which causes an enlarged thyroid gland, tension, swollen eyes, and weight loss (Muldoon *et*

al., 2014). In Graves' disease (GD) and Chronic Autoimmune Thyroiditis (cAIT), T cells and B cells infiltrate the thyroid gland, producing antibodies against clinical signs of hyperthyroidism. T cells in Hashimoto's thyroiditis also cause apoptosis in thyroid follicular cells, eventually leading to the gland's death. Thyroid problems are caused by cytokines, which function throughout the immune system as well as directly attack thyroid follicular cells. They play a critical role in the pathophysiology of autoimmune thyroid disease by being engaged in the induction and effector phases of the immune response as well as inflammation. Multiple cytokines have been found in inflammatory cells and thyroid follicular cells, including IL-1α, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-14, TNF-α, and IFN- γ. T-cell-derived cytokines can damage thyroid cells directly, causing functional problems, as well as induce the synthesis of nitric oxide (NO) and prostaglandin (PG), exacerbating the inflammatory response in AITD. Although the immunological processes involved in the pathogenesis of AITD are closely linked, discrepancies in the picture of the cAIT and GD phenotypes may be attributed to a distinct type of immune response seen in these two counteracting clinical thyroid illnesses (Mikoś *et al*., 2014). In Rheumatoid arthritis, the joint inflammation causes an increase in the thickness of joint cartilage tissues, resulting in swelling and joint pain and possibly damaging the bones themselves (Ostrowska *et al.,* 2018). Many proinflammatory cytokines such as TNF alpha, IL-1, IL-6, GM-CSF, and chemokines such as IL-8 are plentiful in all patients, according to cytokine mRNA and protein analysis in rheumatoid arthritis tissue (Feldmann *et al*., 1996). inflammatory disruption occurs in diabetes Type 1, in which the immune system attacks the insulin-producing cells in the pancreas, which leads to high blood sugar (Donath *et al.,* 2003). In the development of type 1 diabetes (T1D), cytokines play a critical role in coordinating intricate multicellular interactions between pancreatic cells and immune cells. Cytokines with regulatory activities, such as IL-10, TGF-β, and IL-33, are expected to re-establish immunological tolerance and protect -cells. T1D is hypothesised to be triggered and progressed by cytokines such as IL-6, IL-17, IL-21, and TNF-α, which stimulate the development and activity of diabetogenic immune cells (Lu *et al*., 2020). A great amount of data in cancer indicate to pro-inflammatory cytokines as the molecules responsible for metabolic abnormalities associated with cancer-bearing states and cancer development. Pro-inflammatory cytokines like IL-1 and TNF are the most common. The kind of genes activated by these cytokines reflects the inflammation they cause. Cytokines like IL-1 and TNF create chemokines that attract neutrophils in the pathophysiology of carcinogenesis as well as tumour development and dissemination. Neutrophils play an important role in the formation of reactive oxygen species and the development of cancer. The activation of adhesion molecules and

metalloproteinases, both of which provide pathways for tumour invasion, is another feature of pro-inflammatory cytokines. (Dinarello, 2006). In cancer, TNF-α has a role in regulating a number of signalling pathways and affects tumour formation by numerous methods, including contributing to epithelial-mesenchymal transition (EMT) which is an important mechanism in cancer metastasis that enables carcinoma cells to suppress their epithelial features changing to mesenchymal ones. This change allows cells to acquire mobility and the capacity to migrate from the primary site (Chen *et al*., 2017). Also, increase cell proliferation, and speeds up angiogenesis, among others, by binding to TNF- R-1 and TNF- R-2. TNF-overexpression has been discovered in a number of cancers, including prostate cancer, ovarian cancer, liver cancer, and breast cancer (Lan *et al*., 2021). Moreover, in some cases of microbial infections cute inflammation and as a result of substantial and uncontrolled secretion of cytokines leading to septic shock and the possibility of failure of several bodily organs (Feghali & Wright, 1997; Odabasi & Cinel, 2020). The most recognisable example here is COVID-19 disease which infects the upper and later the lower respiratory tracts resulting in pneumonia in most cases and Acute Respiratory Distress Syndrome (ARDS) in approximately 15 % of the cases. The death rate in COVID-19 cases has been linked to the extreme release of pro-inflammatory cytokines "cytokine storm" induced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Odabasi & Cinel, 2020). This cytokine storm leads to worsening of ARDS and widespread damage of the lung tissues resulting in multi-organ failure and death (Ragab *et al.,* 2020; Coperchini *et al.,* 2020).

The reason for the occurrence of immune system disorders is often unknown; however, that may be due to some bacteria and viruses causing changes that confuse the immune system. Here, the immune system cannot differentiate between bacteria or viruses and healthy tissues and begins to fight healthy tissues (Casadevall & Pirofski, 2003; Hooper *et al.,* 2012). The genetic factor is considered one of the most important causes for these diseases, so the body is ready for it. This factor is present since birth in the person genome (Whitacre, 2001). Some other factors may increase the chances of immune disease, including family history; people who have a family history of the immune disease have a higher chance of developing the same immune disease (Benros *et al.,* 2012; Martino & Prescott, 2010). The balance of the "intestine microbiome" is important for the development of the immune system, but also microorganisms defend themself against the body own immune system, which could cause inflammatory distribution (Zheng *et al.,*2020). Some diets are likely to be rich in saturated fats and salts, which are linked to the increase in the incidence of immune diseases (Veldhoen & Brucklacher-Waldert, 2012; Wilck *et al*., 2019). The disease catalyst factor could also occur due to surgeries,

during the postpartum period, psychological conditions or changes in the body hormones percentage, and there are many other factors (Akutsu *et al.,* 2020; Ganio *et al.,* 2020; Sánchez-Maldonado *et al.,* 2019). Inflammatory diseases can affect anyone, and people with these diseases cannot practice their life as required, except when taking drugs with gradually reducing the dose according to the patient's improvement and his response to treatment. However, many inflammatory diseases have no proven pharmaceutical treatment other than early appropriate management strategies by antibiotics, source control, fluids resuscitation and vasopressor agent (Hager *et al.,* 2019; Karbian *et al.,* 2020). Traditionally, synthetic non-steroidal medicines, corticosteroids, synthetic disease-modifying anti-inflammatories, and immunosuppressants have all been used to treat immunological illnesses. However, such medications are frequently linked to side effects such as gastrointestinal issues ranging from moderate to severe dyspeptic symptoms, the development of stomach or duodenal ulcers, bleeding, or perforation. They may also be linked to heart, liver, and renal issues, as well as opportunistic infections and other events that might result in hospitalisation or death. (Russell, 2001; Rainsford, 2007; McCarberg & Gibofsky, 2012; Dinarello, 2011; Li *et al.,* 2017). As TNF-α and IL-1β are generally considered as the pro-inflammatory cytokine's masters and play a crucial role in the pathogenesis of many autoimmune diseases (Mizutani *et al.,* 1997; Soriano *et al.,* 2013; Zbakh *et al*., 2020; Lu *et al*., 2006; Niccolai *et al*., 2020; Mocellin & Nitti, 2008), Novel anti-inflammatory agents with fewer side effects and lower toxicity based on their anti-TNF-α and IL-1β properties are required. These medications are currently being hailed as innovative treatments for illnesses characterised by cytokines associated with chronic inflammation disorders. Brown macroalgae offer natural products that represent a safer with lower cost, as proposed in this study.

1.3.2 Brown seaweed as a source of anti-proinflammatory agents

Since the first identification of immune and inflammatory disorders, research on anti-inflammatory therapy focused on synthetic substances. However, in recent years, more studies have highlighted natural products as safe and abundant alternative sources that can contribute to the pharmaceutical industry of anti-inflammatory agents (Kim, 2010; Yang & Zhang, 2017). Brown macroalgae are well recognised to be a rich source of bioactive compounds reported as anti-inflammatory agents through inhibiting the release of inflammatory mediators. Brown macroalgae represent a vastly untapped resource of fatty acids, which could be more sustainable than other natural resources. For instance, the *Ishige okamurae* rich fatty acids fractions significantly suppress allergic inflammation in human basophilic KU812F cells by reducing the production as well as expression of interleukin IL-4 and IL-13 (Vo *et al*., 2011). Moreover, different types of lipids including phospholipids, non-polar glycerolipids, glycolipids, betaine lipids, sterol and fatty acids of stearidonic acid and eicosapentaenoic acid and arachidonic acid were isolated from the *Undaria pinnatifida* showed inhibitory effects against the inflammatory symptoms of edema, erythema, and blood flow in mouse ear inflammation (Khan *et al*., 2007). The author suggested that arachidonic acid can work as an anti-inflammatory lipid mediator. Phyto-oleic acid nanovesicles made by petroleum ether extract *S. binderi* decreased IL-2, TNF-α, and IL-1β production, improve wound healing rate and elevate serum IL-4 in Wistar rats (Moni *et al*., 2018). Fucosterol from *S. binderi* was found to Suppress COX-2, PGE2, TNF-α, and IL-6 production via the inhibition of NF-κB activation and MAPK group phosphorylation in human lung epithelial cells (Fernando *et al*., 2019). Yang *et al* (2013), isolated Sargachromenol from *S. micracanthum* and found that the lipid demonstrated suppression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), PGE2, NO, COX-2, and iNOS production via inhibition of IkappaB kinase (Iκβ) degradation in LPS-induced RAW264.7. Methanolic extract and its fractions of *Eisenia bicyclis* were found to have strong anti-inflammatory activity. The fraction was purified and was found to contain fucosterol which inhibited t-BHP-induced ROS generation and suppressed the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The author suggested that the anti-inflammatory mood of action might attribute to inhibition of NO and ROS generation and suppression of the NF-κB pathway (Jung *et al*., 2013). Brown seaweed polysaccharides compounds have also been reported to have potent anti-inflammatory in several studies. For instance, fucoidan isolated from *Turbinaria decurrens* reduced the licking time thereby suggesting an anti-nociceptive effect and decreased the size of paw swelling in inflammatory

edema in mice model. Fucoidan showed down regulation of the gene expression of pro-inflammatory mediators such as IL-1β, COX-2 and MMP-9 in fucoidan treated. The anti-inflammatory effect of fucoidan was attributed to its capacity for modulating the levels of enzymatic antioxidants, master regulator NF-κB and pro-inflammatory cytokines (Manikandan *et al*., 2020). Moreover, fucoidans isolated from *Ecklonia cava* significantly inhibited NO production in LPS-induced Raw 264.7 macrophage cells by down-regulating the expression of iNOS, COX-2, and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (Lee *et al*., 2012). However, in a study on low molecular weight fucoidan extracted from New Zealand *Undaria pinnatifida* promoted significant NO release, iNOS expression, and TNF-α and IL-6 secretion in RAW264.7 macrophages in a concentration-dependent manner. It also significantly stimulated the activation of NF-κB and MAPK signalling pathways, and specific inhibitors of NF-κB and MAPK pathways diminished the stimulation, confirming the activation pathways (Bi *et al*., 2018). The polysaccharide Laminarin is abundant in Laminaria and *Saccharina* species and has the potential to be as a useful therapeutic agent with immunostimulatory and anti-inflammatory properties. It is a storage β-glucan which is composed of (1,3)-β-d-glucan and some β-(1,6)-intrachain links and it can represent up to 35% of the dry weight of *Laminaria* and *Saccharina* (Kadam *et al*., 2015). According to O'Shea *et al* (2016). a combination of laminarin and fucoidan exhibit potent anti-inflammatory activities in the gastrointestinal tract following a dextran sodium sulfate (DSS) challenge in pigs. The combination improved body-weight loss, diarrhoeal scores and clinical variables associated with a DSS challenge in pigs, in tandem with a reduction in colonic IL-6 mRNA abundance. The polysaccharide alginic acid isolated from *S. horneri* exhibited suppression of PGE2, TNF-α, IL-6, and IL-1β, and COX-2 production via inhibition of NF-κB (p65) nuclear translocation and MAPK (ERK1/2, JNK, and p38) phosphorylation in CFD (Chinese fine dust)-induced HaCaT keratinocytes (Fernando *et al*., 2018). The same compound was isolated from *S. wightii* and was found to have potent autoinflammatory activity via suppressing the production of COX-2, 5-LOX, MPO, xanthine oxidase (XO), ceruloplasmin, rheumatoid factor, CRP, pro-inflammatory cytokines, and lysosomal enzymes in type-2 collagen-induced rat arthritis (Sarithakumari & Kurup, 2013). A potential anti-inflammatory effect of the Carotenoid fucoxanthin isolated from brown alga *Myagropsis myagroides* was reported by Heo *et al*. (2010), who confirmed that the pigment inhibited the nitric oxide β in LPS induced RAW 264.7 macrophage cells and considerably reduced the production of IL-1β, IL-6, and TNF-α via decreasing mRNA expression levels. In another study by the same author, fucoxanthin was isolated from *S. siliquastrum* and demonstrated suppression of TNF-α, IL-1β, and IL-6, PGE2,

NO, COX-2, and iNOS production in LPS-induced RAW 264.7 (Heo *et al*., 2012). Polyphenols are another group of bioactive potential anti-inflammatory compounds that exist abundantly in and have been isolated from brown seaweeds. For instance, Diphlorethohydroxycarmalol is a phlorotannin compound isolated from *Ishige okamuarae*. It was found to strongly reduce the production of IL-6, in LPS -stimulated RAW 264.7 macrophages. The compound suppressed the phosphorylation and the nuclear translocation of NF-κB and inhibited JAK-signal transducer and activator of transcription (STAT) which are central signalling molecules in the inflammation process induced by LPS (Kang *et al*., 2015). Moreover, Phlorotannin from *S. muticum* showed suppression of ROS production in PMA-induced neutrophils and suppression of PGE2, COX-1, and COX-2 expression in A23187-induced erythrocytes (Casas *et al*., 2016). Phlorotannin subfraction from the Alaskan brown seaweed *Fucus distichus* reduced mRNA expression of acute and chronic inflammatory biomarkers in RAW 264.7 macrophages. Expression of TLR4 and TLR9 were also reduced, suggesting a potential mechanism of anti-inflammatory activity via TLR attenuation (Kellogg *et al*., 2015). Dieckol from *Ecklonia cava* demonstrated inhibition of LPS-induced iNOS and COX-2 protein and mRNA expression, suppression of p-38/ NF-κB pathway and ROS scavenging activity in mouse microglial BV-2 cells (Jung *et al*., 2009). Phlorofucofuroeckol B from *Ecklonia stolonifera* showed inhibition of IκB-α/NF-κB and Akt/ERK/JNK pathways mouse microglial BV-2 cells (Yu *et al*., 2015). As cited above, there are numerous anti-inflammatory activities attributed to primary and secondary metabolites from brown seaweed, where compounds such as polysaccharides, polyphenols and carotenoids hold a special place as they extensively studied and proved to have anti-inflammatory compounds with a wide range of mechanisms in both *in vivo* and *in vitro* studies.

Cystoseira genus abundantly occurs in quality and quantity in the Mediterranean Sea coasts and it is well known to have a high concentration of bioactive compounds (Cecchi & Cinelli, 1992; De Sousa *et al.,* 2017), Despite this, most of them have not yet been investigated for pharmacological and biological activities which could lead to a new promising therapeutic strategy for inflammatory diseases. The genus has been announced in many studies to contain a high content of fatty acids, polyphenols, flavonoids, polysaccharides, peptides, and pigments (Custódio *et al.,*2016; Mhadhebi *et al.,* 2011; De Sousa *et al.,* 2017). However, very few studies have been conducted on the anti-inflammatory activity of these compounds from *Cystoseira*. Also, most studies in the anti-inflammatory activity of *Cystoseira* focused only on crude extracts or fractions and did not intend to purify and identify the active component. For

instance, extracts from the species *C. amentacea* from the Ligurian Sea showed anti-inflammatory properties by inhibiting inflammatory cytokines and mediators such as NO production and IL-1α, IL-6, cyclooxygenase-2 and inducible NO synthase gene expression in RAW 264.7 macrophages (De La Fuente *et al.*, 2021). Phlorotannin rich extracts of *C. tamariscifolia on* the west coast of Portugal showed inhibitory of NO in LPS-stimulated RAW 264.7 macrophage cells (Lopes *et al.*, 2012). Three Tunisian *C. crinita*, *C. sedoides* and *C. compressa* extracts exhibited significant anti-inflammatory activity in a dose dependent manner, by comparison, to reference drugs *in vivo*, using carrageenan induced rat paw oedema assay (Mhadhebi *et al.*, 2014). Chloroform an ethyl acetate and methanol fractions from the Tunisian *C. sedoides* and *C. compressa* exhibited dose-dependent anti-inflammatory activity, comparable with the reference drug acetylsalicylic–lysine, using the carrageenan-induced rat paw edema model (Mhadhebi *et al.*, 2011; Mhadhebi *et al.*, 2012). Fresh and dry extracts of *Cystoseira myrica* from the Egypt Red Sea coast displayed significant depletion in serum TNF-α level -inflammatory actions thioacetamide-induced liver injury in rats (Ahmed *et al.*, 2016). It is clear that several *Cystoseira* bioactive compounds are involved in a variety of immunomodulation activities. Yet some are already exploited, for example, meroterpenoids isolated from *Cystoseira usneoides* from the Mediterranean coast significantly reduced the production of TNF-α, IL-6, and IL-1β, and suppressed the COX-2 and iNOS expression, in LPS-stimulated cells (Zbakh *et al.*, 2020; De Los Reyes *et al.*, 2013). Also, sulphated polysaccharides from three Mediterranean *C. sedoides*, *C. compressa* and *C. crinita*, exhibited significant anti-inflammatory activity in the carrageenan-induced rat paw edema (Ammar *et al.*, 2015). Still, most of the compounds have not been studied in detail. For instance, total lipid concentration ranged from 6.7 in *C. humilis* to 9.4 mg g^{-1} of dry weight in *C. nodicaulis* and 8.4 mg g^{-1} of dry weight in *C. tamariscifolia* (Vizetto-Duarte *et al.*, 2015). *Cystoseira* has one of the highest fatty acid content among brown seaweed (Choudhary *et al.*, 2021). For example, *C. humilis* for example has a high fatty acid content, about 48% compared to other brown seaweeds studied from the Atlantic coast of Morocco, in which PUFAs, linoleic acid and arachidonic acid are abundant (Belattmania *et al.*, 2018). *C. indica* is also rich in fatty acid with 1.23± 0.11 % of dry weight (Kumari *et al.*, 2010). These fatty acids have been shown to exert a multitude of beneficial health via regulating inflammatory activities and decreasing the risk of arthritis, diabetes, and obesity (Fernando *et al.*, 2016; Barbalace *et al.*, 2019). The anti-inflammation activity mechanisms of fatty acids are responsible for gene and cytokine expression regulation among other processes (Robertson *et al.*, 2015). They also function as competitors to the pro-inflammatory metabolism of linoleic acid and as mediators of

prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) inflammation pathways which can be enzymatically converted to bioactive autacoids with inflammatory-resolving properties that compete with and offset inflammation status (McCauley *et al*., 2015). These together suppress the activity of nuclear transcription factors, such as NF-κB, and reduce the production of pro-inflammatory enzymes and cytokines, including COX-2, TNF-α, and interleukin IL-1β (Kang & Weylandt, 2008). Fatty acids have also been associated with anti-inflammatory properties by inhibiting pro-inflammatory cytokines interleukin IL-6 and IL-8 production and by the inhibition of nitric oxide (NO) production (Da Costa *et al*., 2017). However, much has yet to be discovered about the compositional and anti-inflammatory properties of *Cystoseira* bioactive compounds, including fatty acids, and hence their potential as disease-preventing ingredients. Therefore, an evaluation of the anti-proinflammatory activity of *C. tamariscifolia*, *C. crinita* and C. *compressa* extracts and purification of the active compounds in a pharmacological approach should be applied as a pre-clinical stage in the aspect of drug discovery.

As referred above, there are numerous biological activities attributed to primary and secondary metabolites from brown algae. The biological activities assigned to *Cystoseira* are related to both extracts and isolated compounds. Among these bioactivities, the anticancer and anti-inflammatory are especially important, since there is a search for new biological compounds with the potential to replace those used in therapeutics for which there are already many resistances and side effects. Chemotherapeutic strategies currently used to treat different cancers are not entirely effective and are often associated with severe side effects that can significantly reduce the quality of life of the patient. Compared to traditional chemotherapy, natural compounds can overcome drug resistance with lower side effects. Thus, much research is being dedicated to finding novel compounds that show clinical efficacy with reduced side effects, with much of this research concentrated on natural products from brown algae. Since *Cystoseira* lives in an environment that is subject to relatively extreme environmental changes, the production of these bioactive substances may change based on the surrounding biotic and abiotic factors that appear in seasons, and this may add unique properties or increase the yield of the bioactive compounds, which may give them an advantage as anti-cancer agents. Therefore, this study is interested in evaluating the effect of the seasonal changes on *Cystoseira* biologically active compounds composition and their anti-cancer activity. Moreover, as anti-inflammatories and immunosuppressants medications are frequently linked to side effects that might result in hospitalisation or death, the search for alternatives from natural products that

are safe and abundant is being intensifying. The abundance of bioactive compounds in *Cystoseira*, which have proven to be effective as anti-inflammatory agents when isolated from other types of brown algae or some terrestrial plants, opens the door for studying the anti-inflammatory activity of *Cystoseira* extracts, especially for samples collected from the Libyan coast, which did not previously undergo purification and isolation or identification.

1.4 Aims of the current study

This research study aimed to investigate the proximate biochemical composition and *in vitro* cytotoxic activity of brown seaweed *C. tamariscifolia* gathered from the U.K and *C. crinita* and *C. compressa* species collected from Libya. The intention was to investigate the effects of the extraction method and seasonality on *C. tamariscifolia* extracts biochemical composition using colourimetric assays and cytotoxic activity on human cancer cell lines *in vitro* using MTT and trypan blue assays. To expand the knowledge of the cytotoxic effect mechanism of the active extracts, we performed fluorescent staining assays (chapter 3). It was interesting to continue investigating *Cystoseira* extracts for anticancer activity via immunomodulation methods as we aimed to test our *Cystoseira* extracts for stimulating the immune cell to fight cancer. However, surprisingly the extracts showed exciting results on the inhibition of cytokine release. We then decided next to investigate the anti-inflammation activity of *Cystoseira* spp. extracts. We aimed to explore the anti-proinflammatory effect of the most exciting extracts of *Cystoseira* spp on the inhibition of IL-1β and TNF-α release from stimulated M1 macrophage-like cells (reported in chapter 4 and chapter 5).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2. General materials and methods

2.1 Collection of *Cystoseira* spp.

Cystoseira tamariscifolia was collected by hand from the shallow subtidal at Hannafore Point, Cornwall, UK (DD N 50.342234, W 4.453528) in four seasons while C. *crinita* and C. *compressa* were collected from the east coast of Libya in summer and dried in the shade. The collected samples were transferred to the laboratory in polythene bags kept in an icebox. On arrival, the samples were rinsed with distilled water, cleaned of adhering debris and epiphytes. The samples were then freezed at −20°C and then freeze-dried using Edwards super Modulyo freeze dryer.

2.2 Solvent extraction

Extracts were made in chloroform, methanol (100% and 70%) and water. Fifty grams of freeze-dried samples were mixed with solvents (1:10, w/v) and then homogenised for 2 min with an IKAT10B Ultra-Turrax disperser at 24 °C. The extract was then stirred for 3min, centrifuged (5000×g, 10min, room-temperature [RT]), and the supernatants were recovered. The extracts were dried at 40 °C under vacuum. Solvents were used to re-suspend all extracts, and then, the extracts were stored at − 20 °C for biological activity screening (Vizetto-Duarte *et al.,* 2016).

2.3 Total polyphenol content (TPC)

The Folin–Ciocalteu (F–C) colourimetric technique (Velioglu *et al.,* 1998) was used to determine total phenolic concentrations (TPC). Briefly, 5 µL of the extracts at 0.1, 1 and 10 mg mL^{-1} were mixed with 100 µL of 10-fold diluted F–C reagent, incubated at RT for 5 min and mixed with 100 µL of sodium carbonate (75 g L^{-1}). Following a 90-min incubation period at RT, absorbance was measured at 725 nm on a microplate reader (Omega, BMG Labtech). The concentration of TPC was calculated as phloroglucinol equivalents (PGE) utilising a calibration curve prepared with phloroglucinol standard solutions and is expressed as mg PGE g^{-1} dry weight.

2.4 Total flavonoid content (TFC)

Flavonoid concentrations were quantified according to the method described by Ahn *et al.* (2007), with modifications. Briefly, 50 µL of 2% (w/v) aluminium chloride–ethanol solution was added to 50 µL of the extracts at 0.1, 1 and 10 mg mL^{-1}. After 1 h at RT, the absorbance was measured at 420 nm on a microplate reader (Omega, BMG Labtech). Quercetin was used as a standard, and results are expressed as mg of quercetin equivalents (QE) g^{-1} dry weight.

2.5 Total polysaccharides content

Total polysaccharide concentrations were measured by the phenol–sulphuric acid method (Masuko *et al.,* 2005). Fifty microlitres of samples at the concentrations 0.1, 1 and 10 mg mL^{-1} was added to 150 µL of sulphuric acid (96% reagent grade). The mixture was incubated in a 96-well plate floating on a water bath at 90 °C for 5 min. Thirty microlitres of 5% phenol were added to the mixture for another 5 min in the water bath. The plate was then floated on cold water for an additional 5 min to cool, and the absorbance was measured at 490 nm on a microplate reader (Omega, BMG Labtech). Glucose was used as a standard, and results are expressed as mg of glucose equivalents (G) g^{-1} dry weight.

2.6 Total protein

The BCA assay was carried out to determine total protein concentrations using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) in a 96-well-plate format. The assay mixture contained 100 µL of the reagent, and 100 µL of the sample, crude extracts at concentrations 0.1, 1 and 10 mg mL^{-1}. A standard curve with serial Bovine Serum Albumin (BSA) solutions (ranging from 0.1 to 100 µg mL^{-1}) was used for calibration. The mixture was incubated at 37 °C, and absorbance was measured at 560 nm on a microplate reader (Omega, BMG Labtech).

2.7 Cell line maintenance and culture

The culturing of the human leukaemia cancer cell lines THP-1, HL60 and mantle cell lymphoma MCL were as a suspension, and the human prostate cancer cell line (PC3) was as a monolayer. Cells were divided every 3-4 d and were used regularly between passages 10-35. The cells were cultured in RPMI 1640 complete growth medium supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine and maintained at 37 °C, under 5% CO_2 in a humidified incubator.

2.8 Cell vitality assay

The cells were incubated at a density of 2×10^5 cells $well^{-1}$ in a 96-well microplate with four types of *Cystoseira* crude extracts namely, 100% methanol, 70% methanol, water and chloroform extracts at final concentrations of 2.5, 7.5, 25, 75, 150, 250 and 750 µg mL^{-1} or fractions for 72 h at 37 °C. In the case of the PC3 cells were seeded in a 96-well plate and incubated for 24 h at 37°C with 5% CO_2 saturation. The next day the serial of the concentrations of the extracts were applied to the PC3 and incubated for 72 h. In positive control treatment, cells were treated for 72 h with the same solvent as in the extract treatment at the same concentrations. The seeded cells were then incubated for 2 h at 37 °C with 30µl of thiazolyl

blue tetrazolium (2 mg mL^{-1} in PBS). The resulting formazan crystals were solubilised in 150 µL DMSO, and absorbance was read at 540 nm using a microplate reader (Omega, BMG Labtech). The IC_{50} values were calculated from a sigmoidal dose-response curve of the data generated in SigmaPlot v. 13.0.

2.9 Determining the cell vitality of cancer cells lines using Trypan Blue Stain on Automated Cell Counter

Cancer cell lines in suspension at 2×10^5 cell $well^{-1}$ were exposed to methanol extracts of *C. tamariscifolia*, C. *crinita* and *C. compressa* at the final concentration of 250 µg $mL^{-1}$for 72h. Cell lines were then mixed 1:1 with a 0.4% solution of Trypan Blue Stain (Cat. No. T10282), and a 10 µL sample was added to a chamber slide and inserted into a Countess® II FL Automated Cell Counter (Cat. No. AMQAF1000). Cells were allowed to settle for 30 seconds after loading the sample to help ensure a uniform focal plane and accurate counts. The slide was inserted into the counting instrument's sample port to initiate autofocus. Graphs were selected and the gating feature was used to measure the size and to identify the cell population of interest.

2.10 Fluorescent staining for HL60 and THP-1 cells

THP-1 and HL60 were seeded at 10^6 cells mL^{-1} in 24-well culture plates and treated with 100% methanol extracts of *C. tamariscifolia* for 48 h. Cells were centrifuged and suspended in PBS. Propidium iodide (5 µg mL^{-1}in PBS; Sigma Aldrich) was used for staining cells for 5 min at room temperature in the dark, and images were captured using a Nikon fluorescence microscope.

2.11 Heat-killing *Escherichia coli* strain K12

Escherichia coli strain K12 were grown overnight in the laboratory, and then spun down at 6000 rpm for 5 minutes, and washed 3 times with Phosphate-Buffered Saline (PBS). *E. coli* were heat-killed at 70°C for 10 minutes. They were washed and re-suspended in those particular macrophages medium and stored in aliquots at -20°C until the experiment time.

2.12 Limulus Amebocyte Lysate (LAL) endotoxin detection assay

LAL assay kit from Genscript Company was performed to test gram-negative bacterial endotoxin that could be in the seaweed extracts. Briefly: Carefully, 100 µl of standard or test sample was dispensed into endotoxin-free vials and mixed for 30 seconds. Then, 100 µl of reconstituted LAL was added to each vial and swirled gently. Samples were incubated at 37 °C

for 20 minutes on a heating block, and then 100 µl of reconstituted chromogenic substrate solution was added to each vial and incubated at 37 °C for 6 minutes. After that, a stop solution (500 µl) was added to each vial and swirled gently, followed by adding 500 µl of reconstituted Colour-stabilizer 2 to each vial and mixed well. Finally, 500 µl of reconstituted Colour-stabilizer was added to each vial and mixed well. The mixtures were transferred to 96 well plate, and the absorbance of each reaction was measured at 545 nm.

2.13 THP-1 Cell differentiation using phorbol 12-myristate 13-acetate (PMA)

Phorbol 12-myristate 13-acetate (PMA) from Sigma-Aldrich was used to differentiate THP-1 leukaemia cell line to M1 macrophage-like, according to Daigneault *et al.*, 2010 Method. A concentration of 500 ng mL^{-1} of PMA was added to each well in a 24 well-plate at a density of 5 x 10^5 cells mL^{-1}. After four days, the media were washed. On day five, the macrophage-like cells were ready for carrying out further experiments.

2.14 Macrophage-like cells stimulation by Lipopolysaccharides (LPS), Lipoteichoic acid (LTA) heat-killed and live *E. coli* K12 and exposition to the *Cystoseira* methanol extracts

On day five, human macrophage-like cells were stimulated in the presence of LPS from *E. coli* K12 at the concentration of 1 µg mL^{-1} and LTA from *Staphylococcus aureus* at the concentration of 5 µg mL^{-1} and also, both live and heat-killed *E. coli* K 12 at a final concentration of 10^9 cell mL^{-1}. After 2 hours of the stimulating, the stimulated macrophages were exposited to methanol extracts of *C. tamariscifolia*, *C. crinita* and *C. compressa* at final concentrations of 25, 75, 150 and 250 µg mL^{-1} and incubated at 37°C under 5% CO_2 in a humidified incubator. After 18 hours, the supernatants were collected, and the live *E. coli* K12 were counted again, and TNF-α and IL-1β release were estimated by ELISA.

2.15 Detection of *Cystoseira* immunomodulatory activity on TNF-α and IL-1β release from M1 macrophage-like using ELISA.

To detect TNF-α release from stimulated and treated human macrophage-like cells, an ELISA kit was obtained from R&D System Company. TNF-α capture antibody was diluted to the working concentration in PBS. Immediately a 96-well microplate was coated with 100 µL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature. Each well was aspirated and washed three times with wash buffer with complete removal of the liquid at each time. Plates were blocked by adding 300 µL of reagent diluent, which is a solution of Bovine Serum Albumin (BSA) in PBS, to each well and incubated at room temperature for a minimum of 2 hours. After washing the reagent diluent, 100 µL of

sample or standards were added and covered with an adhesive strip and incubated for 2 hours at room temperature. With repeating the washes after each step, 100 μL of the detection antibody were added to each well and incubated for 2 hours at room temperature and then covered with a new adhesive strip. Working dilution of streptavidin-HRP followed by substrate solution was added to each well for 20 minutes for each step at room temperature and covered the plate and incubate away from direct light. 50 μL of stop solution was added to each well with gentle mixing, and finally, the plates were read at wavelength 450 nm using a microplate reader (Omega, BMG Labtech).

2.16 Bacteria culture preparation

Müeller-Hinton agar from Sigma-Aldrich medium was used to grow the bacteria. This medium supports the adequate growth of the tested bacteria, which gives obtained zones in a disk diffusion test that meet the acceptable quality control limits. Thirty-eight grams of dehydrated agar was suspended in a litre of distilled water and then autoclaved. After autoclaving, the media was immediately cooled in a 45°C incubator. The cool media were poured into 90 mm diameters plastic, flat-bottomed Petri dishes to roughly 4 mm depth and then were allowed to cool to room temperature.

2.17 Procedure for performing the disc diffusion test

Human pathogenic bacteria colonies with the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 5 ml of PBS. A spectrophotometric device was used to obtain a suspension containing approximately 1 to 2 x 10^8 CFU mL^{-1} for human pathogenic positive and negative gram bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli.* Sterile cotton swabs were dipped into the well mixed suspensions and then were rotated several times on the entire sterile surface of the agar with repeating this two more times in different directions. Finally, swabbed agar was allowed to absorb the applied bacteria before applying the seaweed extracts disks. Whatman filter paper disks filled with 5 µl of solvent as a negative control and *Cystoseira* crude extracts at concentrations of 1, 3, 6, 10, 30, 100 mg mL^{-1} were applied to the culture as well as a disk of the antibiotic gentamicin at the concentration of 10µg. After 24 hours of incubation, each plate is examined. The diameters of the zones of complete inhibition, including the diameter of the disk were measured.

2.18 *Cystoseira* crude extract profiling and fractionation by High-Performance Liquid Chromatography (HLPC)

HPLC analysis was performed using an Ultimate 3000 HPLC system from Thermo-Fisher combined with an auto-sampler and variable UV wavelength detector. The chromatography was performed on BDS HYPERSIL C18 column from Thermo-Fisher with 4.6 mm internal diameter, 250 mm length, and 5 µm particle size. Chromeleon version 7.2 software provided by the supplier was performed to control the instrument and analyse the data. *Cystoseira* crude extracts were profiled using water (Solvent A) and methanol (Solvent C) as mobile phases. Gradient elution was performed by varying the proportion of solvent C to solvent A. The gradient elution was changed from 10 % to 90% (solvent C: solvent A: 10: 90) in 65 min. Another 10 min, before the injection of another sample. The flow rate was 1 ml/min, and the column temperature was 28°C. The injection volume was 20 µl for the profiling process, and the ultra-violate UV detector was adjusted at wavelengths (254, 272, 280 and 310 nm).

2.19 Initial HPLC fractionation of methanol extract of *Cystoseira* spp

An initial fractionation process in order to purify the responsible compounds for the anti-proinflammatory effect of *C. tamariscifolia* and *C. crinita* methanol extracts was performed, similar chromatographic conditions that in HPLC extracts profiling were used to separate the crude methanol extracts of both *C. tamariscifolia* and *C. crinita* bioactive compounds by HPLC. Here, a higher concentration of the crude extract 100 mg mL^{-1} and a bigger injection volume of 100 µL was applied to obtain a higher volume of fractions at high concentration. The fractions were collected manually every 10 min during the retention time, and 6 fractions for each species were obtained and next concentrated using sample concentrator Biotage -V10- and then resolved in methanol solvents to obtain the concentration of 250µg mL^{-1}. Fractions then were then kept in the freezer at -20 until the biological experiments.

2.20 Statistical analysis

Anti-proinflammatory experiments were repeated at least three times, and the results were presented as (mean± SD). Student T-test was performed in excel to examine the significant differences between groups at p-value 0.05.

2.21 Sample preparation for LC/UV (DAD) and LC/ESI-MS analysis

The sample of 50µl of active anti-inflammatory fraction from *C. crinita* was vacuum-dried then re-solvated in 20µl 1: 1 MeOH: H_2O for the analysis by LC/UV (DAD) and +ve and –ve ion LC/ESI-MS detection using standard NMSF methods were used for the analysis.

2.22 LC/UV (DAD) analysis

Agilent 1100 HPLC System with Diode Array Detector was used for LC/UV analysis. The HPLC Analysis Conditions were Waters XBridge C18 column with dimensions of 3.5 µm x 2.1 mm x 150 mm, and the Guard column was XBridge C18 3.5 µm x 2.1 mm x 10 mm. The Flow rate was 200 µl/min (Agilent) and 150 µl/min (Dionex). Mobile phases were A = H_2O and B = MeOH and the 5µl full loop injection. Mobile Phase Gradient was 2% B at 0 time (mins) to 90% 45 time(mins). UV-Vis wavelength 254, 272, 280, 310 nm with BRandwidth from 4 to 16 and reference 360 at 100. The Diode Array DAD spectrum brand was from 190-400nm.

2.23 LC-ESMS analysis

Dionex Ultimate 3000 and LTQ Orbitrap XL combined with an atmospheric pressure ionisation (API)from Thermo-Fisher was used for LC/MS for the characterisation of the interest compounds. As the chemical properties of the putative components of interest in the sample are unknown, the analysis was carried out under similar optimised conditions in positive and negative ion mode. Operating parameters of the API interface were optimised in the full scan mode (m/z 160-1600) with a resolution of 30,000. Optimum conditions were as follows: Capillary gas temperature 285°C (+ve mode) and 325°C (-ve mode), solid needle probe voltage 4 kV (+ve mode) and -4.2 kV (-ve mode), Sheath gas flow rate 15 ml/min, and auxiliary gas flow is 2 units in both modes, capillary voltage 49 V (+ve mode) and -30 (-ve mode) and tube lens voltage 150V (+ve mode) and -100V(-ve mode).

CHAPTER 3

THE BIOCHEMICAL COMPOSITION AND CYTOTOXIC ACTIVITY OF THE BROWN ALGA *CYSTOSEIRA* SPP. AGAINST CANCER CELL LINES

3.1 Introduction

Cancer is a major cause of death worldwide, and it is likely to remain a major affliction to patients and a challenge to healthcare services. Over 200 different types of cancer and approximately fifty per cent of people worldwide will get cancer, including prostate, leukaemia and lymphoma in their lifetime (Nascimento-Gonçalves *et al.,* 2018; Nordqvist, 2017; Hallek, 2019). One of the most common treatments for cancer is still chemotherapy (Coffelt & de-Visser, 2015; Senthebane *et al.,* 2017). For instance, cisplatin is a platinum-based medication, specifically cis-diamminedichloroplatinum(II), that is used to treat a variety of solid tumours, including testicular, ovarian, head and neck, colorectal, bladder, and lung cancers. Cisplatin acts against cancer in a variety of ways; it causes DNA lesions, activates the DNA damage response, and induces mitochondrial death. Despite a high proportion of early responses, cisplatin therapy frequently results in chemoresistance, which leads to therapeutic failure (Galluzzi *et al*., 2012). Carboplatin is a second-generation platinum compound that has been recommended for the treatment of ovarian and lung cancers, as well as squamous cell carcinomas of the head and neck and seminomas. It appears to have a qualitative range of action comparable to that of cisplatin. As with cisplatin, carboplatin interacts with nucleophilic sites on DNA, forming intrastrand and interstrand crosslinks, as well as crosslinks between DNA and proteins (Adams *et al*., 1989). However, carboplatin has a much different hazard profile than cisplatin, with nephrotoxicity, neurotoxicity, and ototoxicity occurring seldom with carboplatin. As with cisplatin, nausea and vomiting occur in a significant proportion of patients following carboplatin administration, however, symptoms are often delayed several hours and are mild to moderate in intensity. Dose-limiting nausea and vomiting are uncommon with carboplatin. Carboplatin's dose-limiting hazard is myelosuppression, characterised by severe thrombocytopenia and, less frequently, leukopenia, which may be more severe in elderly individuals, those with renal impairment, or those who have previously had chemotherapy (Wagstaff *et al*., 1989; Bisch *et al*., 2018). Etoposide is a semi-synthetic chemical obtained from the North American mayapple, *Podophyllum peltatum*, and the Indian species *Podophyllum emodi* plants. It is used to control and cure many malignancies, including testicular, prostate, bladder, stomach, and lung cancer (Sinkule, 1984). It belongs to the class of drugs known as topoisomerase II inhibitors. It acts predominantly during the late S and G2 stages of the cell cycle. Topoisomerase II simultaneously cuts both strands of the DNA helix. During the replication process, it produces and repairs double-stranded DNA breaks. Etoposide poisons topoisomerase II cleavage complexes, impairing the reaction's second stage, DNA re-

ligation. The etoposide-topoisomerase II complex initiates a mutagenesis and cell-death cascade, which is most effective in tumour cells with increased topoisomerase II enzyme levels (Montecucco *et al*., 2015). Etoposide is also an effective chemotherapeutic agent for other cancers, including refractory paediatric neoplasms, hepatocellular carcinomas, and acute nonlymphocytic leukaemia, but is ineffective against malignant melanoma, colorectal cancer, head and neck cancer, prostatic carcinomas, ovarian carcinomas, and non-small cell lung cancer. It is also harmful to the gastrointestinal tract, causing alopecia and gastrointestinal poisoning. Nausea, vomiting, and stomatitis are all symptoms of gastrointestinal poisoning. (Marigny *et al*., 2005; Ehl *et al*., 2018). Chemotherapeutic strategies currently used to treat different cancers are not entirely effective and are often associated with severe side effects that can significantly reduce the quality of life of the patient. Thus, much research is being dedicated to finding novel compounds that show clinical efficacy with reduced side effects, with much of this research concentrated on natural products, including those from marine algae (Anand *et al.,* 2016). Bioactive compounds from brown seaweeds have been highlighted for their importance as anticancer agents (Ashwini *et al.,* 2016). Most of these bioactive compounds are generally safe and are abundant in brown seaweeds, which make them a promising source of therapeutic compounds (Zubia *et al.,* 2009). A wide range of these bioactive ingredients has been reported to have anti-tumour activity. For instance, the phlorotannin dieckol from *Ecklonia cava* showed anticancer activity via inducing apoptosis and inhibited the growth of human pancreatic cancer cells PANC-1, inhibition of non-small-cell lung cancer (Xu *et al*., 2021). Fractions with high sulfated polysaccharide levels extracted from *S. pallidum* exhibited significant cytotoxicity against a variety of cancer cell lines, including lung adenocarcinoma A549, hepatocellular carcinoma HepG2 and gastric cancer MGC-803 cell lines (Ye *et al*., 2008). The antitumour activity of the fractions was inversely proportional to the sulphate level. The author suggests that high sulphate concentration and low molecular weight are beneficial for its anticancer action. Sulfated polysaccharides derived from *Dictyopteris delicatula* and *Dictyopteris polyodioides* were shown to have an anticancer effect in cervical HeLa and human skin malignant melanoma RPMI-7951 cancer cell lines (Magalhaes *et al*., 2011; Sokolova *et al*., 2011). The authors examined the specific mechanisms behind these anticancer effects, which included tumour angiogenesis, immune system regulation, cell cycle arrest, and death. The fatty acid palmitic acid purified from *Turbinaria ornata* exhibits an inhibitory effect on HT-29 human colon cancer cells *in vitro* with an IC_{50} value of 36.04 μg mL^{-1}. Apoptosis associated factors like apoptosis induction were observed and cell cycle analysis exposed that cell were inhibited in the G0/G1 phase. (Bharath *et al*., 2021). The carotenoids fucoxanthin

extracted from *Laminaria Japonica* showed to have anticancer activity against lung cancer cells PC9. This effect may be due to inhibition of tumour cell proliferation and activation of apoptosis (Ming *et al*., 2021). Given the wide range of compounds in brown seaweeds that they and initial evidence on the anticancer activities of those compounds, the *Cystoseira* genus represent a valuable and rich source of these compounds making the genus a promising strategy to discover a novel anticancer agent. *Cystoseira* is a rich source of bioactive compounds with unique structural features and is well known to show cytotoxic activities against cancer cell lines (Li *et al.,* 2011; Alghazeer *et al.,* 2016; De Sousa *et al.,* 2017; Gutiérrez-Rodríguez *et al.,* 2017; Abdelhamid *et al*., 2019). Moreover, several studies have demonstrated that the production of the bioactive compounds varies seasonally (e.g., Stengel *et al.,* 2011) and that changes in biotic and abiotic factors between seasons and locations could have an effect (Celis-Plá *et al.,* 2016; Zatelli *et al.,* 2018). However, as far as we are aware, while concentrations of secondary metabolites have been measured, no assessment of seasonal variations in cytotoxicity against cancer cell lines has been undertaken. Here, we investigate the seasonal variation of the chemical composition and the anticancer activity of *C. tamariscifolia* collected from southwest England. We also examine the cytotoxic activity of Mediterranean *C. crinita* and *C. compressa* against the cancer cell lines for any potential locational effect. Four different cancer cell lines, HL-60, THP-1, PC3 and REC-1, representing two leukaemia-derived lines, a prostate cancer line and mantle cell lymphoma, respectively, were treated with increasing concentrations of four types of extracts of *C. tamariscifolia* (in four seasons) *C. crinita and C. compressa* and cell vitality was assessed via the MTT and trypan blue assays. We show that extracts from *C. tamariscifolia, C. crinita* and *C. compressa* showed a considerable amount of both primary and secondary metabolites. Moreover, extracts from *C. tamariscifolia, C. crinita* demonstrated potent cytotoxic activities in cancer cell lines; however, C. *compressa* showed relatively lower cytotoxic activity against cancer cell lines. We also show extracts from *C. tamariscifolia* harvested in the summer and spring contained higher levels of primary and secondary metabolites. The result also shows that the cytotoxic activity of the bioactive compounds of *C. tamariscifolia* displays seasonal variability ranging from complete cell death to undetectable depending on the season. We thus suggest that when collecting marine algae from temperate seas in order to search for bioactive compounds, a consideration of seasonality is made, and samples are collected during different seasons to maximise the possibility of finding bioactive compounds.

3.2 Results

3.2.1 The biochemical composition of *Cystoseira crinita* and *Cystoseira compressa*

Phytochemical analysis of the extracts of *C. crinita* and *C. compressa* from the Eastern coast of Libya are profiled in the table. (3.1). The tested extracts showed that the most abundant constituents of the *C. crinita* were Total polyphenols Content (TPC) was ranging between 150 mg g^{-1} D.W. in absolute methanol extracts and just around 25 mg g^{-1} D.W. in chloroform extracts. Total polysaccharides content was fluctuating between 20 and 50 mg g^{-1} D.W. throughout the types of solvent extracts. The crude extract of *Cystoseira crinita* showed a relatively high value of total protein in the methanol at a concentration of roughly 13 mg g^{-1} D.W., while chloroform extract was lacking protein. In *C. compressa,* polyphenols were the most abundant compounds in 70% methanol extracts with a concentration of approximately 74.71 mg g^{-1} D.W. In contrast, a low level of total polysaccharide has been detected in all solvent extracts. Protein in *C. compressa* also was not present in chloroform extracts.

Table 3.1 The bioactive compound compositions of the brown seaweed *Cystoseira* spp. Collected in the U.K. and Libyan coasts. *D.W.:* dry weight. Total polyphenol Content: milligram phloroglucinol acid equivalent per gram dry weight; Total flavonoid Content: milligram Quercetin equivalent per gram dry weight. Total polysaccharide: milligram Glucose equivalent per gram dry weight; Total protein: milligram bovine serum albumin equivalent per gram dry weight. Values are presented as mean ± S.D. (n = 3).

The bioactive Components.(mg/g DW)		**Extracts**			
		100%MeOH	**70%MeOH**	**Water**	**Chloroform**
Total polyphenol Content	*Crinita*	153.03 ± 15.1	95.68 ± 6.28	102 ± 11.38	26.86 ± 4.94
	compressa	58.70 ± 0.62	74.71 ± 6.12	41.98 ± 8.29	33.35 ± 3.97
Total flavonoid Content	*Crinita*	57.51 ± 3.88	35.55 ± 5.54	11.25 ± 2.02	47.22 ± 2.28
	compressa	10.22 ± 0.72	10.67 ± 1.18	6.21 ± 0.96	14.43 ± 0.25
Total polysaccharide	*Crinita*	47.85 ± 6.17	18.35 ± 3.62	20.28 ± 4.17	17.47 ± 1.05
	compressa	0.73 ± 0.17	2.31 ± 0.88	5.06 ± 0.31	1.95 ± 0.35
Total protein	*Crinita*	12.63 ± 0.71	6.96 ± 0.36	3.93 ± 0.63	-
	compressa	10.50 ± 0.19	6.73 ± 0.01	6.37 ± 0.29	-

3.2.2 The cytotoxic activity of the Mediterranean brown alga *Cystoseira crinita* and *C. compressa* against cancer cell lines

Four different cancer cell lines, HL-60, THP-1, PC3 and REC-1, representing two leukaemia-derived lines, a prostate cancer line and mantle cell lymphoma, respectively, were treated with increasing concentrations of four types of extracts of *C. crinita and C. compressa* and cell vitality was assessed via the MTT assay, fig (3.1, 3.2). Cytotoxic activity results showed that all types of extracts of the two species could inhibit cell growth effectively, especially the methanol extract of *C. crinita,* which showed high cytotoxic activity against HL60, THP-1 and REC-1 cell with IC_{50}= 85.315 ± 17.12, 103.35 ± 10.30 and 108.886 ± 5.82 µg mL^{-1} respectively. In contrast, against the PC3 cell line, the cytotoxic activity of extracts of *C. crinita* was slightly low. In the case of *C. compressa*, chloroform extracts showed the most reduction in cell vitality, especially against THP-1 and PC3 cell lines.

Cell vitality using MTT assay: cell lines vs all season extracts of *C. crinita*

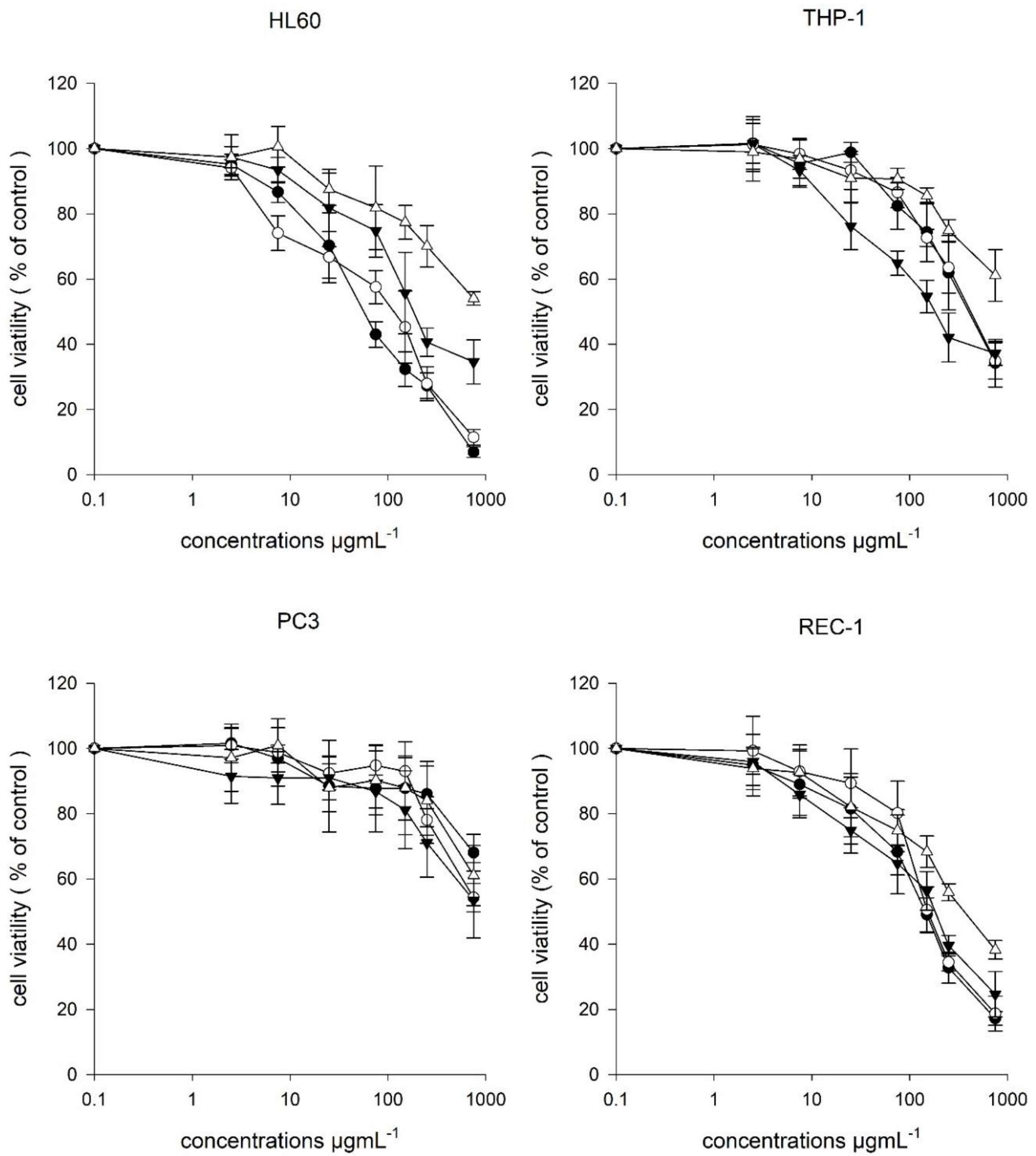


Figure 3.1 Dose-response curves for cell vitality in cancer cell lines. HL60, THP-1, PC3 and REC-1 A288 cells were exposed for 72 h at 37 °C to crude extracts of *C. crinita*. Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).

Cell vitality using MTT assay: cell lines vs all season extracts of *C. compressa*

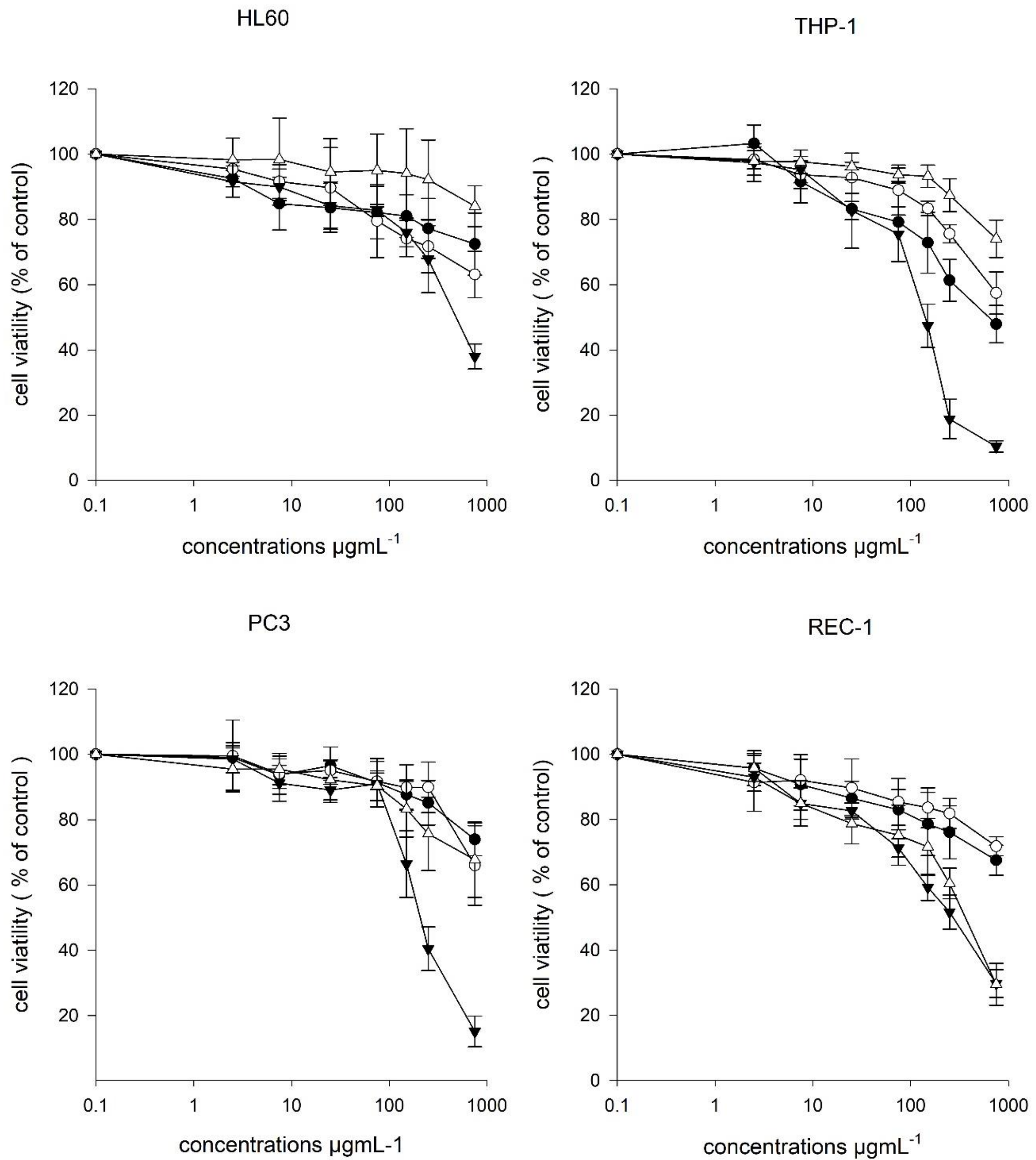


Figure 3.2 Dose-response curves for cell vitality in cancer cell lines. HL60, THP-1, PC3 and REC-1 A288 cells were exposed for 72 h at 37 °C to crude extracts of *C. compressa*. Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).

3.2.3 Effects of extraction method and season on the biochemical composition of *Cystoseira tamariscifolia* extracts

To assess the biochemical composition of *C. tamariscifolia* extracts from the south-western coast of England, various extraction solvents were applied to material collected in all four seasons, and the concentrations of primary and secondary metabolites were measured (Table 3.2).

100% methanol extracts of *C. tamariscifolia* contained the highest yields of primary and secondary metabolites. Polyphenol content was found to be higher in 100% methanol extracts with a concentration of approximately 100 mg g^{-1} D.W., while chloroform extracts showed the highest content of flavonoids with up to 45 mg g^{-1} D.W. Polysaccharide content ranged from 10 and 45 mg g^{-1} D.W. in the water and 100% methanol extracts, respectively (table 1).

The spring and summer extracts generally had higher concentrations of metabolites, while in autumn, we recorded the lowest concentration of total polyphenols and flavonoids. *C. tamariscifolia* crude extracts had low protein content compared with the previous bioactive compounds. The highest level of protein was in the autumn in both 100% methanol and water extracts with concentrations of roughly 19 and 6 mg g^{-1} D.W., respectively, while the chloroform extract could not be used due to interference with the assay.

Table 3.2 The bioactive compositions of the brown seaweed *C. tamariscifolia*. Collected from Hannafore Point, The south-western coast of Britain. *DW:* dry weight. Total polyphenol Content: milligram phloroglucinol acid equivalent per gram dry weight; Total flavonoid Content: milligram Quercetin equivalent per gram dry weight. Total polysaccharide: milligram Glucose equivalent per gram dry weight; Total protein: milligram bovine serum albumin equivalent per gram dry weight. Values are presented as mean ± S.D. (n = 3).

The bioactive Components (mg/g DW)	Season	Extracts			
		100%MeOH	70%MeOH	Water	Chloroform
Total polyphenol Content	Summer	102.23±1.85	57.70±2.06	83.24±1.03	41.99±0.90
	Autumn	71.27±4.16	9.67±1.27	20.61±3.07	7.81±0.31
	Winter	71.16±4.40	31.20±1.19	14.62±0.91	48.89±1.91
	Spring	85.46± 2.7	62.35±1.86	46.29±0.28	68.75±2.79
Total flavonoid Content	Summer	22.87±0.80	5.55±0.46	8.56±0.71	22.27±0.66
	Autumn	27.86±1.20	3.26±0.27	5.99±0.99	16.69±0.52
	Winter	35.23±1.03	5.66±0.91	4.89±0.28	45.19±2.12
	Spring	25.54±0.5	6.69±0.97	4.80±0.12	49.21±4.83
Total polysaccharide	Summer	48.84±3.66	8.16±0.40	14.95±1.85	42.84±3.84
	Autumn	31.10±5.80	11.19±0.96	10.25±1.58	27.81±0.71
	Winter	18.04±2.96	3.92±0.33	3.12±069	25.78±8.74
	Spring	39.11±1.46	19.30±2.26	16.02±040	26.75±0.29
Total protein	Summer	9.28±0.28	8.10±0.59	7.18±0.63	-
	Autumn	19.57±1.42	3.31±0.81	13.51±1.08	-
	Winter	9.95 ± 0.36	5.95 ± 0.31	3.28 ± 0.36	-
	Spring	11.17± 0.12	5.36± 0.43	3.7± 0.07	-

3.2.4 Extraction method and seasonality impact the anticancer activity of *C. tamariscifolia.*

To evaluate the impact of extraction method and seasonality on the potential cytotoxic activity of *C. tamariscifolia*, four cancer cell lines, HL-60, THP-1, PC3 and REC-1, were treated with increasing concentrations of *C. tamariscifolia* extracts and cell vitality was assessed via the MTT assay. Cells were treated with extracts from all four seasons extracted via all four extraction regimes separately (Figures 3.3, 3.4, 3.5 and 3.6), and IC_{50} values for cell vitality were calculated (Table 3.2).

There were some interesting effects of the solvents used for extraction. Generally, 100% methanol and chloroform extracts had the most cytotoxic effect on the three cell lines tested, with some differences between them (Table 2). The 100% MeOH extract was approximately 17 times more cytotoxic in HL-60 compared to water extracts, while both 100% methanol and chloroform extracts had almost identical activity in HL60, THP-1 and REC-1. Extracts made in water were the most cytotoxic in PC3 cells (Figure 2) with IC_{50} values approximately 2-5 fold higher than for the other solvents, whereas water extracts were much less cytotoxic than the other solvents for HL-60, THP-1 and REC-1 cells, being between roughly 2-130 fold less cytotoxic (Figures 1, 3). In addition to the extraction method effect, there was a clear seasonality to the cytotoxic effects on the three different cell lines. Summer and spring extracts showed the most potent cytotoxic effect on THP-1, HL-60 and REC-1 cells with approximately eight times more cytotoxicity compared to autumn and winter extracts. There was, however, an exception in that winter extracts were also highly cytotoxic against the prostate cancer cells PC3.

HL-60 and REC-1 cells showed the greatest susceptibility to *C. tamariscifolia* extracts with a mean IC_{50} over all seasons and extracts of 80.61 ± 21.74 µg mL^{-1} and 82.31 ± 9.67 µg mL^{-1}, respectively, while the similar THP-1 model showed a mean IC_{50} over all seasons and extracts of 199.78 ± 37.23 µg mL^{-1} and PC3, 162.15 ± 36.11 µg mL^{-1}. The most cytotoxic effects were seen from the summer extractions in 100% and 70% MeOH and 100% chloroform on HL-60 cells (IC_{50} values; 2.32 ± 0.21, 7.34 ± 0.30 and 7.92 ± 0.12 µg mL^{-1} respectively; Figure 1). These values show that the cytotoxic potency of these crude extracts is very high against REC-1 and HL-60 cells and is between approximately 17 and 62 fold higher than the corresponding effects on THP-1 and PC3 cells, respectively.

Table 3.3 IC_{50} values (µg mL^{-1}) for extracts on HL60, PC3 and THP-1 cell lines. Cells were exposed for 72 h to crude extracts of *C. tamariscifolia* collected in all seasons. IC_{50} values were calculated by sigmoidal dose-response of the data using SigmaPlot v. 13.0.

		Extracts			
Season	cells	70% MeOH	100% MeOH	Chloroform	Water
Summer	HL60	7.33 ± 0.30	2.32 ± 0.21	7.91 ± 0.12	293.54 ± 25
	PC3	452.01 ± 20	40.09 ± 3.40	112.28 ± 11	24.88 ± 0.21
	THP-1	116.48 ± 15	134.68 ± 24	60.35 ± 0.98	64.72 ± 6.2
	REC-1	49.16 ± 7.4	5.10 ± 0.36	6.04 ± 0.9	165.44 ± 9.7
Autumn	HL60	110.17 ± 9.6	110.43 ± 9.8	46.25 ± 39	221.51 ± 19
	PC3	469.76 ± 31	64.72 ± 5.3	263.59 ± 24	50.42 ± 5.6
	THP-1	236.74 ± 24	262.62 ± 21	396.04 ± 54	24.62 ± 4.1
	REC-1	104.01±10.41	30.16 ± 6.9	69.85 ±8.1	250.14 ± 22.9
Winter	HL60	39.37 ± 3.2	74.86 ± 0.51	24.63 ± 0.13	149.93 ± 0.20
	PC3	168.27 ± 15	105.60 ± 12	149.88 ± 0.41	38.69 ± 2.7
	THP-1	196.66 ± 22	342.39 ± 22	467.11 ± 27	444.29 ± 45
	REC-1	45.66 ± 1.5	32.80 ± 3.7	26.43 ± 3.1	688.20 ± 115.9
Spring	HL60	24.29 ± 0.27	24.28 ± 0.20	24.46 ± 0.19	128.38 ± 17
	PC3	190.75 ± 19	110.83 ± 15	99.35 ± 8.7	228.39 ± 22
	THP-1	74.98 ± 0.45	24.80 ± 0.31	74.84 ± 0.30	169.13 ± 16
	REC-1	27.86 ± 3.84	19.89 ± 3.2	24.70 ± 4.4	67.15 ± 7.5

Cell vitality using MTT assay: HL60 vs all season extracts of *C. tamariscifolia*

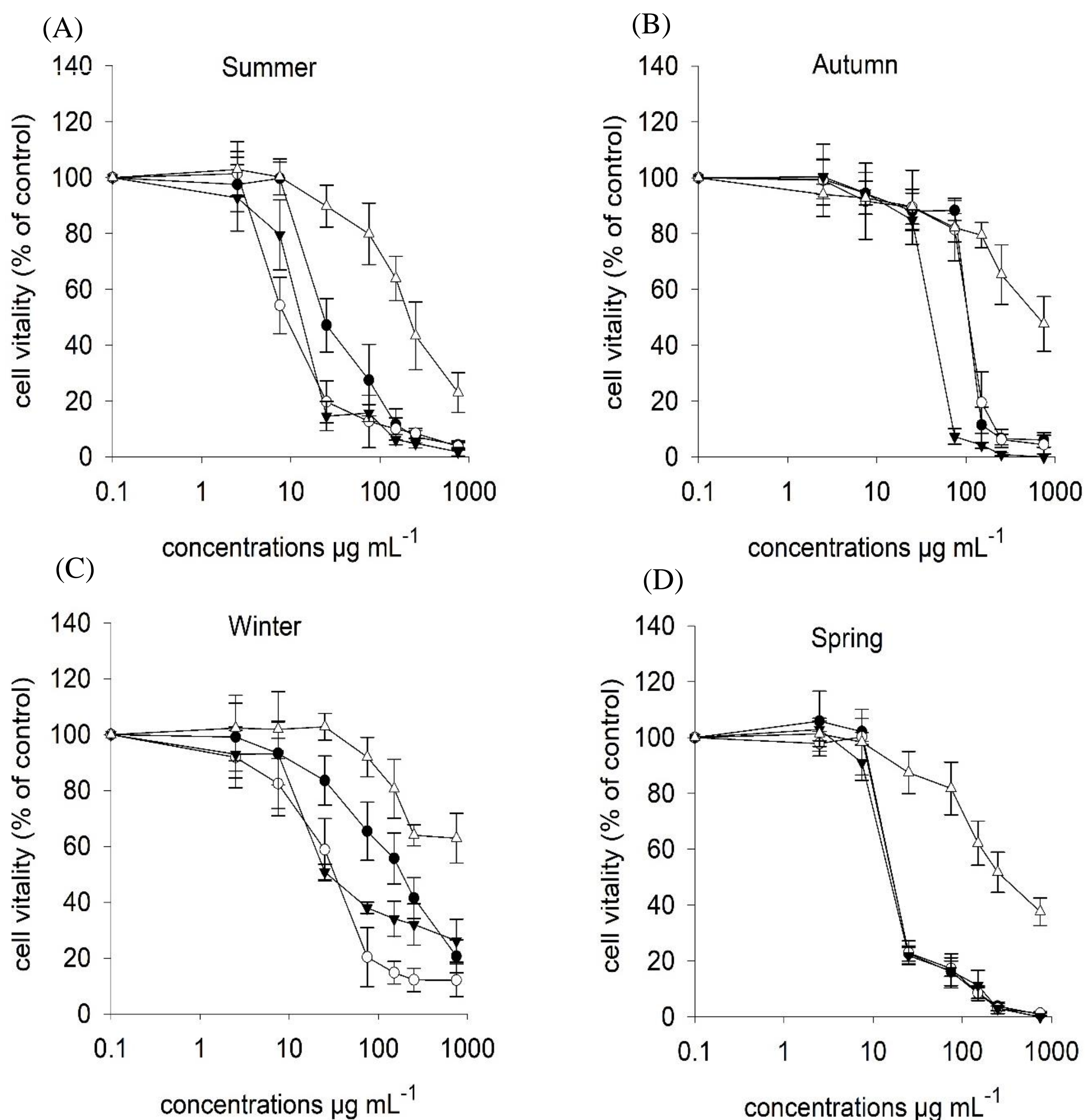


Figure 3.3 Dose-response curves for cell vitality in leukaemia cancer cells. HL60 cells were exposed for 72 h at 37 °C to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.3: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).

Cell vitality using MTT assay: PC3 vs all season extracts of *C. tamariscifolia*

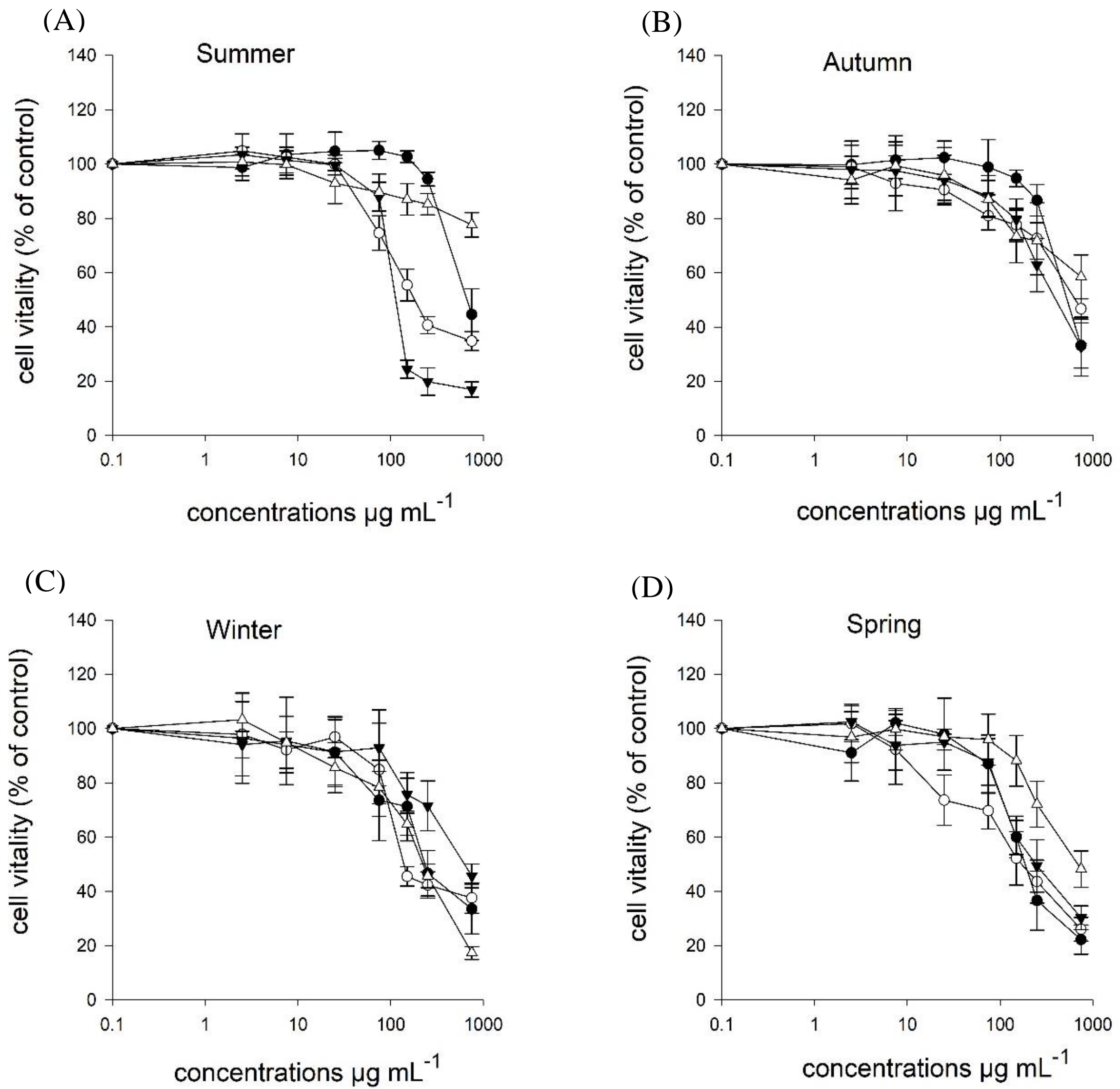


Figure 3.4 Dose-response curves for cell vitality in prostate cancer cells. PC3 cells were exposed for 72 h at 37 °C to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.4: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).

Cell vitality using MTT assay: THP-1 vs all season extracts of *C. tamariscifolia*

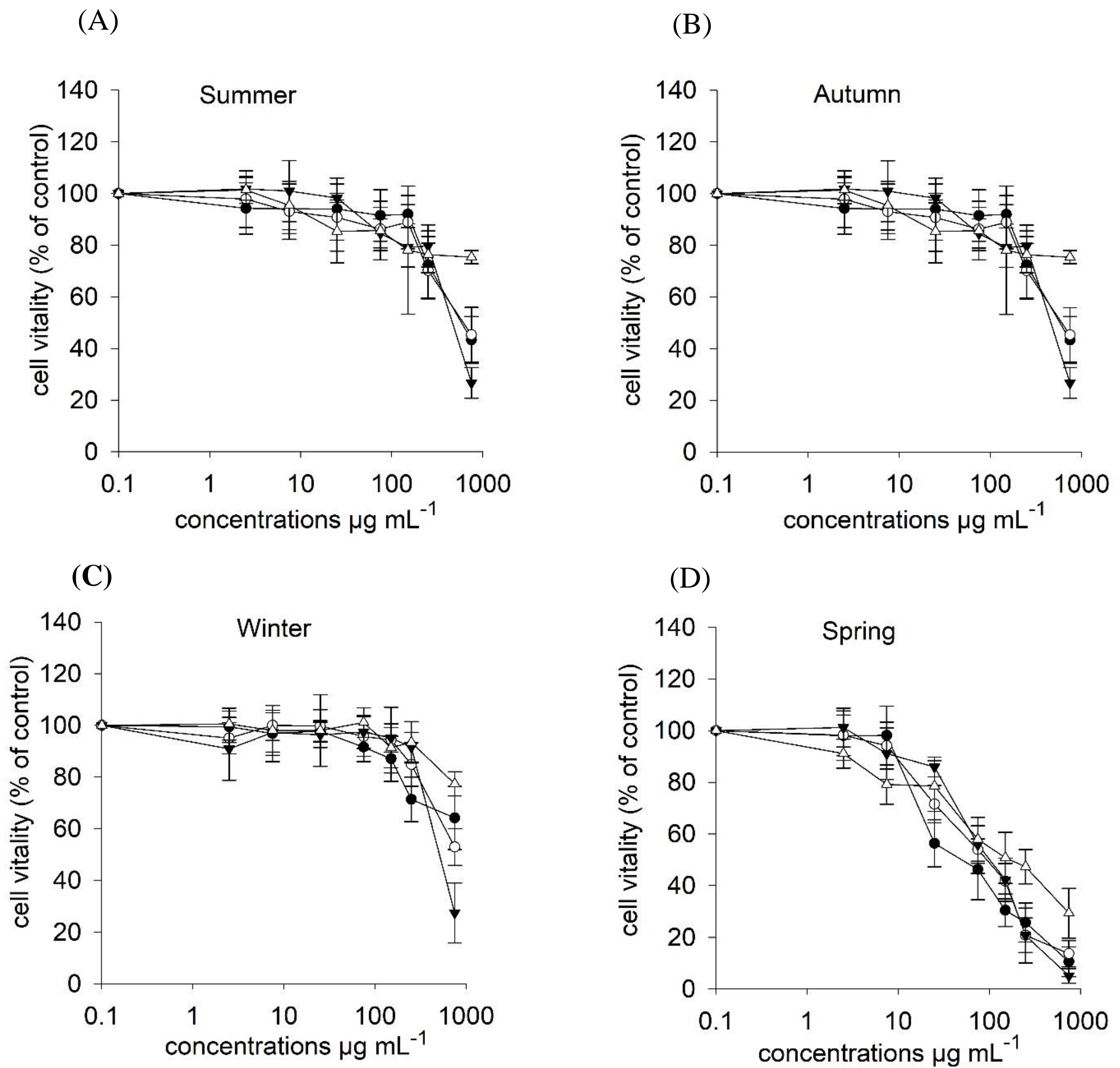


Figure 3.5 Dose-response curves for cell vitality in leukaemia cancer cells. THP-1 cells were exposed for 72 h at 37 °C to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.5: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).

Cell vitality using MTT assay: REC-1 vs all season extracts of *C. tamariscifolia*

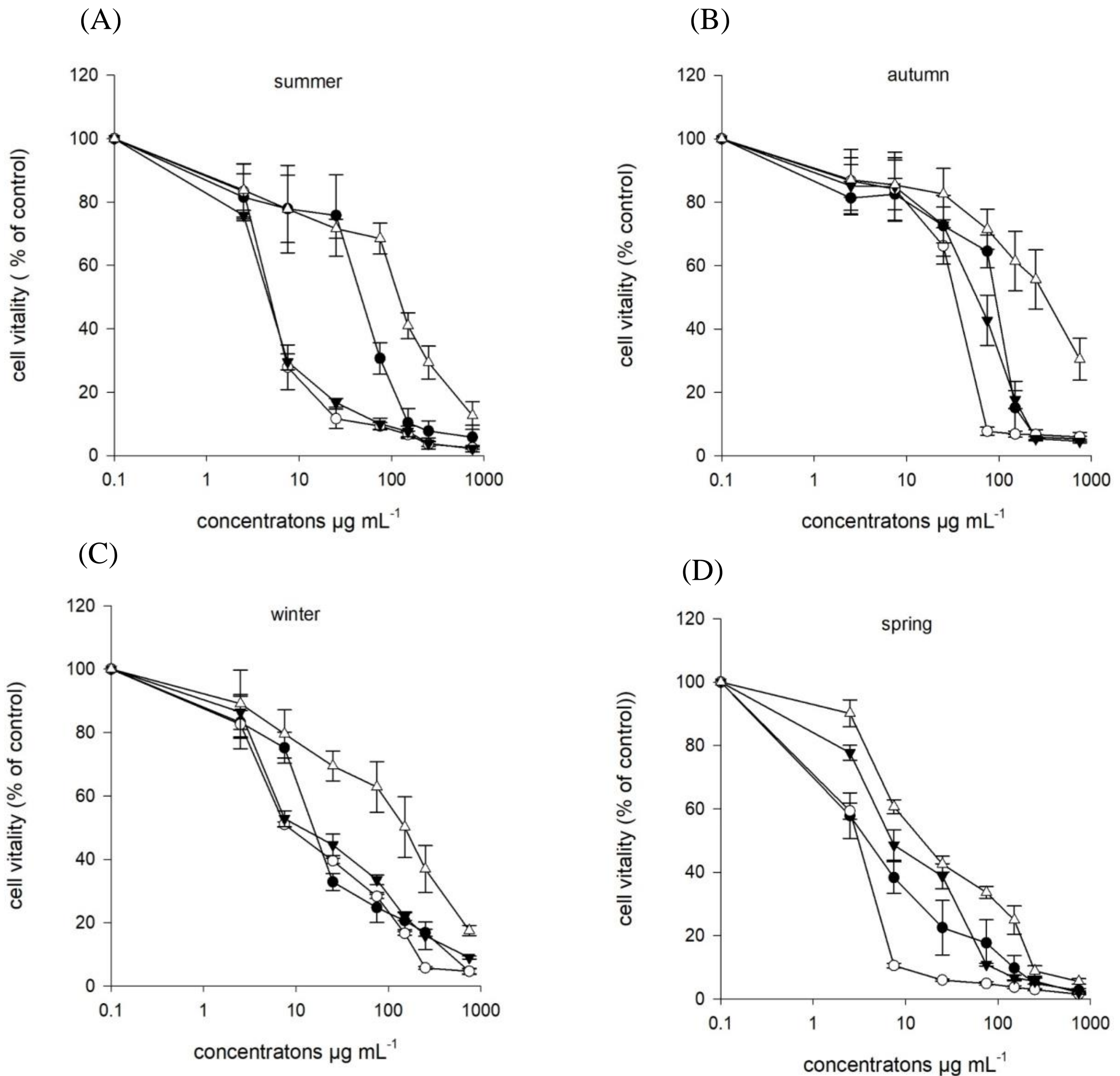


Figure 3.6 Dose-response curves for cell vitality in mantle cell lymphoma. REC-1 A288 cells were exposed for 72 h at 37 °C to crude extracts of *C. tamariscifolia* collected in all seasons (figure 3.6: A, B, C and D). Black circles - 70% MeOH, white circles - 100% MeOH, chloroform - black triangles, white triangles - water. (mean ± SD; n=3).

3.2.5 The suggested correlation between *Cystoseira tamariscifolia* polyphenols and the cytotoxic activity against three cancer cell lines

To explore the effect of the concentration of secondary metabolites and the cytotoxic activity of extracts of *C. tamariscifolia*, we performed a scatter plot to examine the relation between the concentration of polyphenols and the IC_{50} values, as shown in fig 3.7 (A, B, C). All cells showed positive correlation between the concentration of polyphenoles and the cytotoxity activity of extracts. The scatter graphs showed that the increase in total polyphenols level led to a decrease in the IC_{50} values" which means an increase the cytotoxicity effect". The plots showed clear trend and negative correlation between TPC and IC_{50} values against THP-1. However, in case of HL60 and PC3 there was weeker correlation where there were outliner values as the extraction method has an impact on total polyphenols in some events.

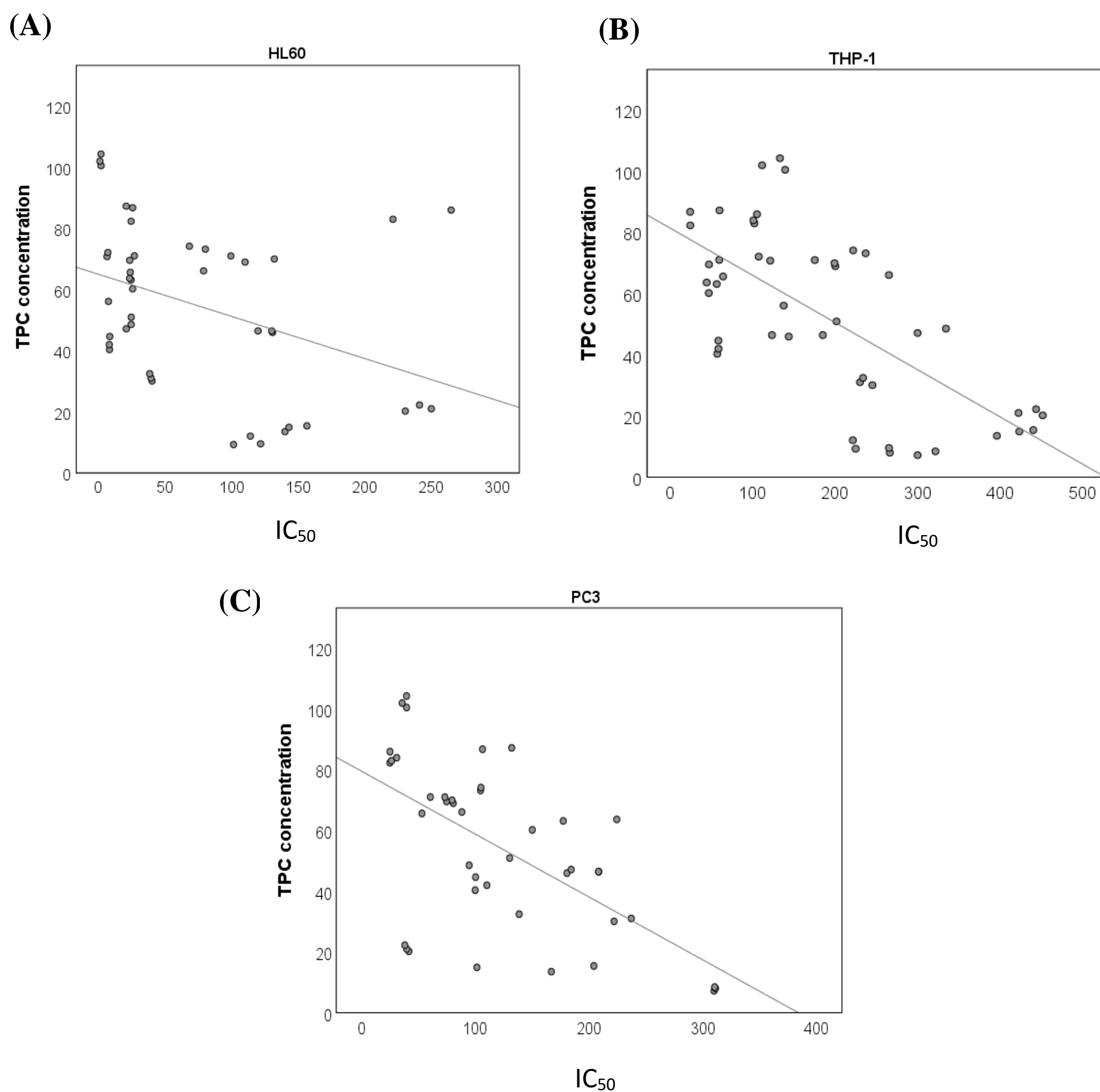


Figure 3.7 Scatter plots show relationships between the concentration of TPC extracted from *C. tamariscifolia* and IC_{50} values data from HL60 (A), THP-1(B) and PC3(C) cytotoxic assay. Graphs show a negative correlation between TPC concentration and IC_{50} values and therefore positive correlation between the concentration of TPC and the cytotoxicity effect of the extracts.

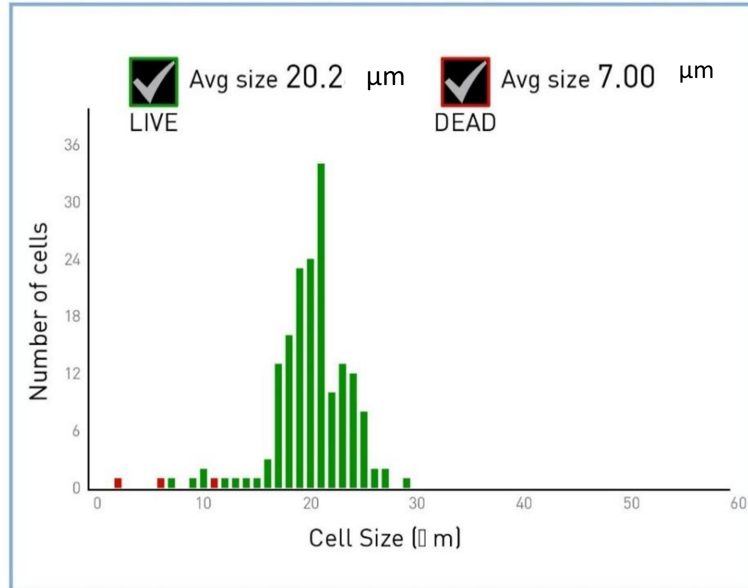
3.2.6 Methanol extracts of *Cystoseira* cytotoxicity activity against cancer cells lines using Trypan Blue Stain on Automated Cell Counter

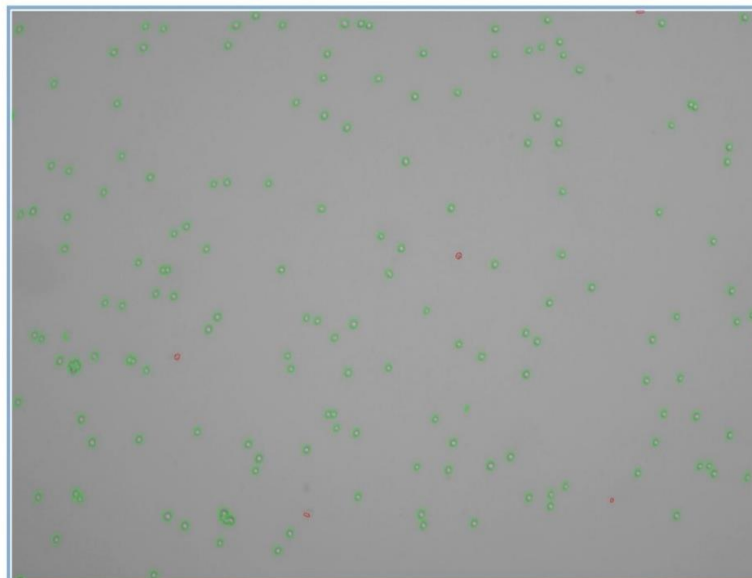
To emphasis the cytotoxic activity of the methanol extracts of *C. tamariscifolia*, *C. crinita* and *C. compressa* at the final concentration of 250 μg mL^{-1} against HL60 and THP-1 cell lines, cell vitality tests were performed using the trypan blue exclusion method. Counting and size measuring were carried out on the Countess® II FL counter. Trypan blue staining data showed high cytotoxic activity of the methanol extracts of *Cystoseira* spp. against both HL60 and THP-1 cell lines after 72h of treatment. As shown in figure 3.8 (A, B, C and D), viable cells with an intact plasma membrane exclude trypan blue dye and appaerd in green coulour in both graphs and pictures, whereas damaged cells become stained, with particularly strong staining in the nucleus and apapered with red coulor. Methanol extracts of *C. tamariscifolia, C. crinita* and *C. compressa* significantly decreased the live cells count in HL60 to 7%, 12% and 12% cells in a population respectively compared to control taken as 97% live cells. The extracts also induced a significant fall in live cells count of THP-1 cell lines to 4%, 16% and 18% cells in a population respectively compared to control taken as 92% live cells, see fig 3.9 (A, B, C and D). Moreover, at the final concentration of 250 μg mL^{-1}, the extract of the three *Cystoseira* species extracts significantly reduced HL60 and THP-1 cells size to 95% and 94%, which markedly affect the cell vitality of treated cells compared to control alone.

Cell vitality using Trypan blue assay: HL60 vs 100% MeOH (control)

(A)

		Concentration
Total		1.02×10^6/mL
Live	97%	9.91×10^5/mL
Dead	3%	2.93×10^4/mL

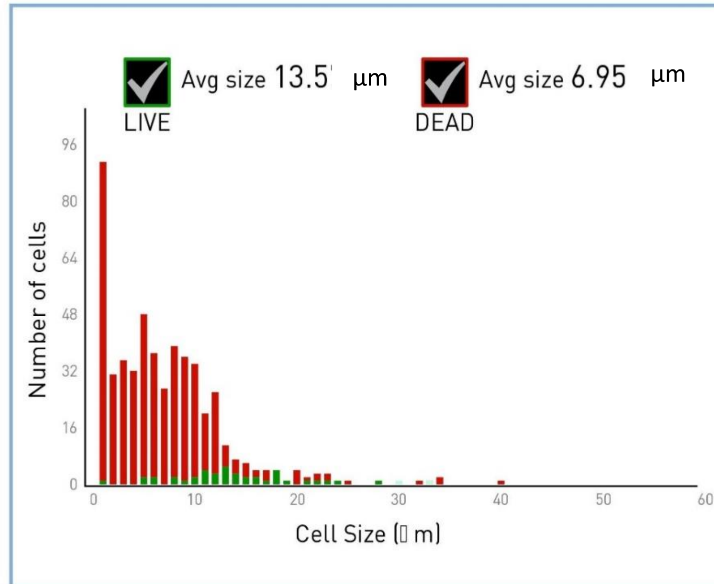




Cell vitality using Trypan blue assay: HL60 vs 100% MeOH extract of *C. tamariscifolia*

(B)

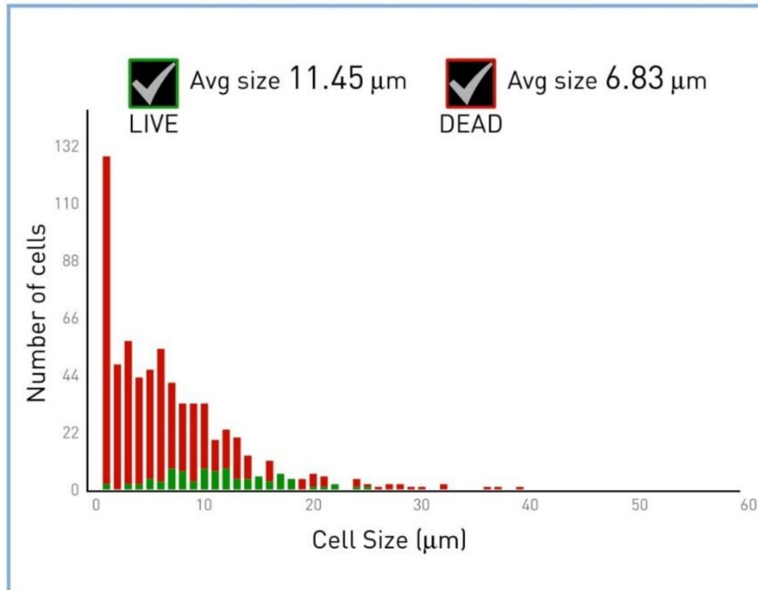
		Concentration
Total		3.23×10^6/mL
Live	7%	2.35×10^5/mL
Dead	93%	2.99×10^6/mL

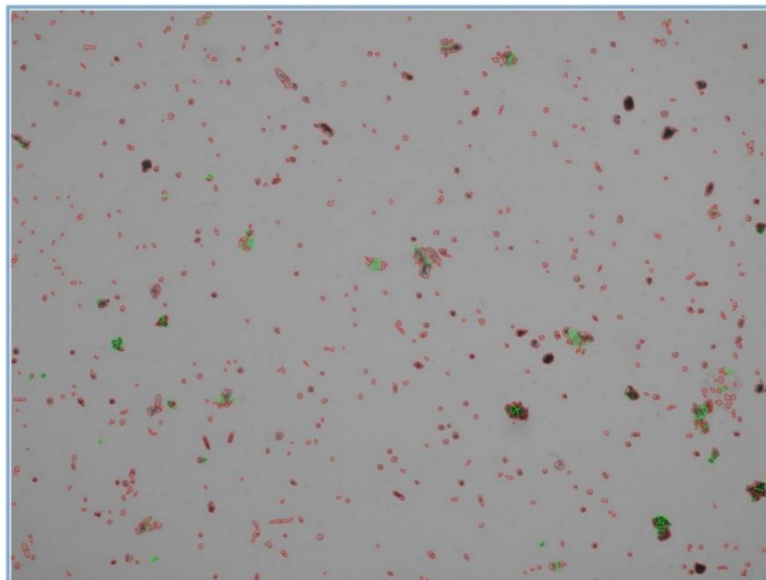


Cell vitality using Trypan blue assay: HL60 vs 100% MeOH extract of *C. crinita*

(C)

		Concentration
Total		4.29×10^6/mL
Live	12%	5.04×10^5/mL
Dead	88%	3.79×10^6/mL

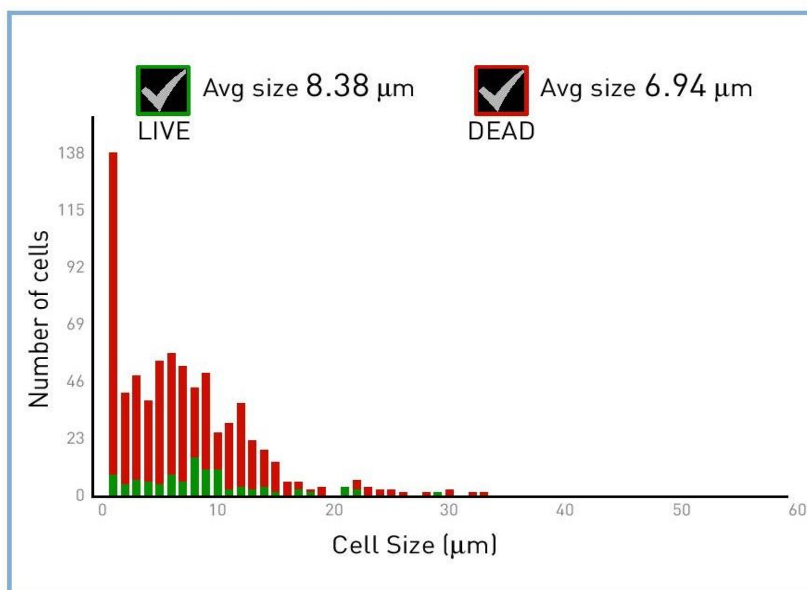




Cell vitality using Trypan blue assay: HL60 vs 100% MeOH extract of *C. compressa*

(D)

	Concentration	
Total		4.67×10^6/mL
Live	12%	5.57×10^5/mL
Dead	88%	4.12×10^6/mL



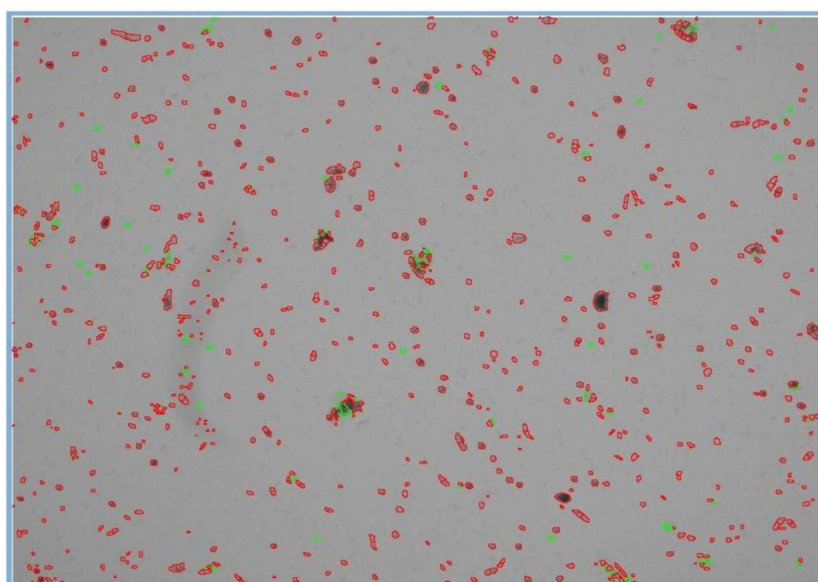
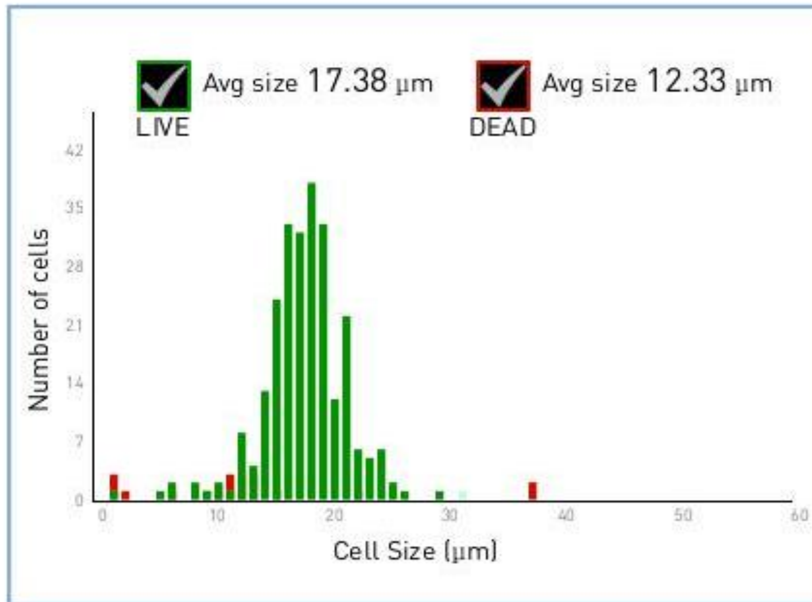


Figure 3.8 Vitality assay via cell staining using the LIVE/DEAD /Cytotoxicity Kit. HL60 cell line exposed to *C. tamariscifolia, C. crinita* and *C. compressa* and incubated for 72h at 37°C. Treated cell lines and control stained with diluted Trypan blue stain and calculated with the Countess II FL Automated Cell Counter instrument equipped with standard Light Cubes. The histogram and the picture show the counts of live cells in the population, which fluoresce green, and dead cells, which fluoresce red.

Cell vitality using Trypan blue assay: THP-1 (control)

(A)

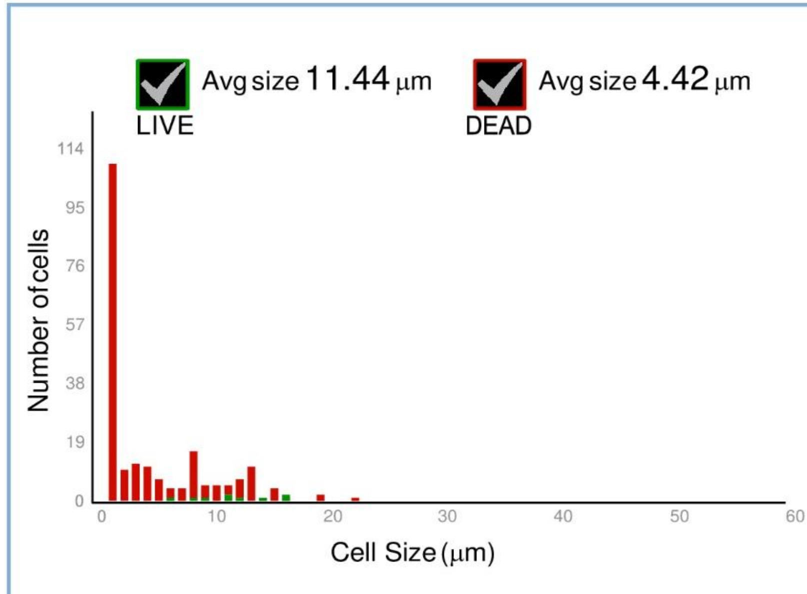
		Concentration
Total		1.59 x 10^6/mL
Live	92%	1.47 x 10^6/mL
Dead	8%	1.23 x 10^5/mL



Cell vitality using Trypan blue assay: THP-1 vs 100% MeOH extract of *C. tamariscifolia*

(B)

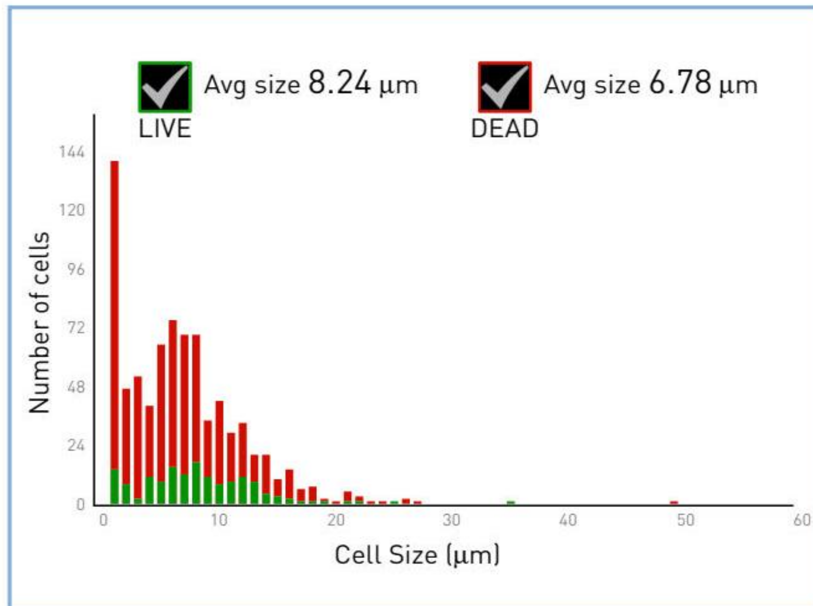
		Concentration
Total		1.31×10^6/mL
Live	4%	5.28×10^4/mL
Dead	96%	1.26×10^6/mL

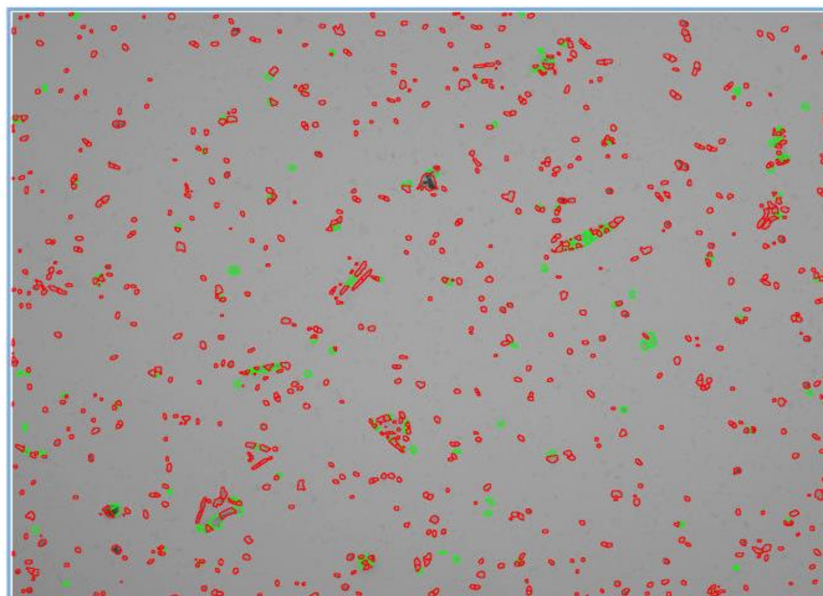


Cell vitality using Trypan blue assay: THP-1 vs 100% MeOH extract of *C. crinita*

(C)

		Concentration
Total		5.52×10^6/mL
Live	16%	8.91×10^5/mL
Dead	84%	4.63×10^6/mL





Cell vitality using Trypan blue assay: THP-1 vs 100% MeOH extract of *C. compressa*

(D)

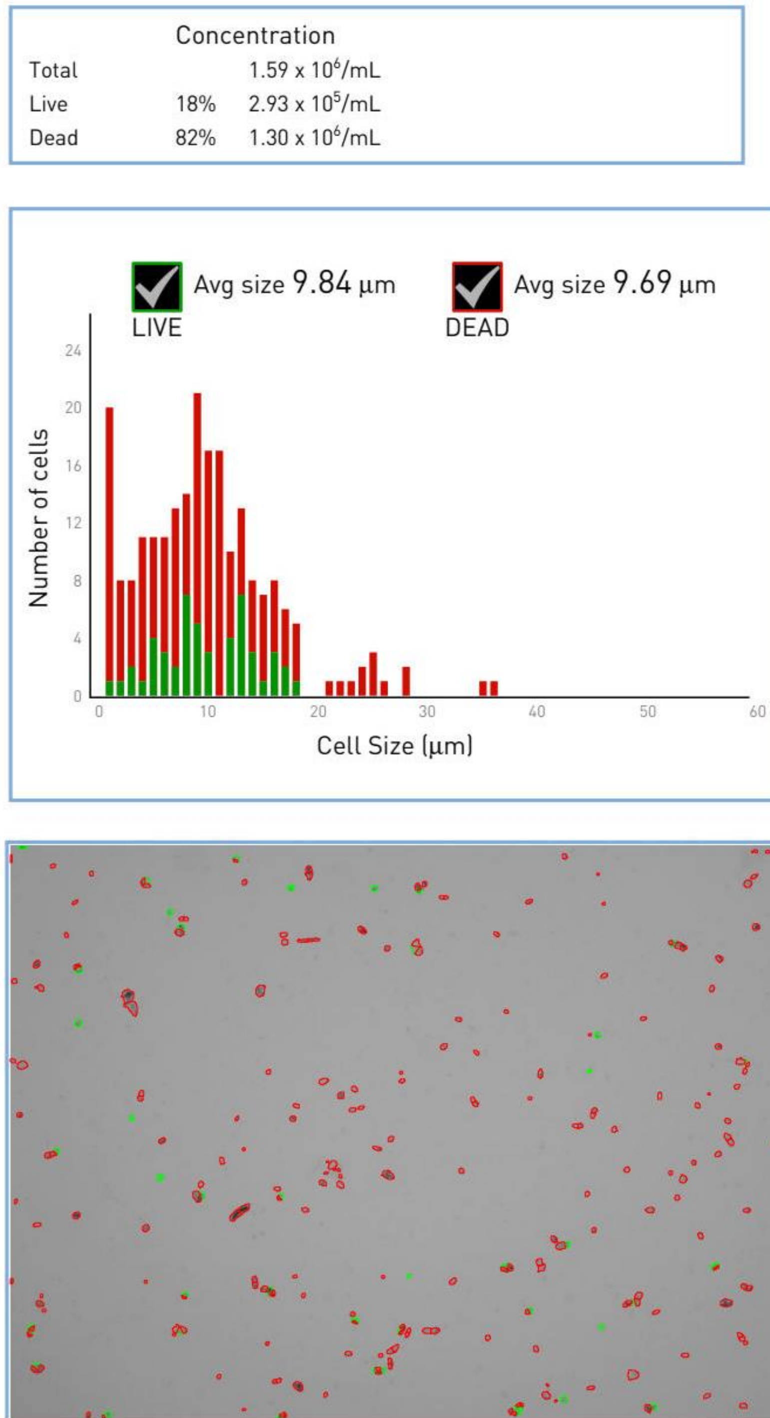
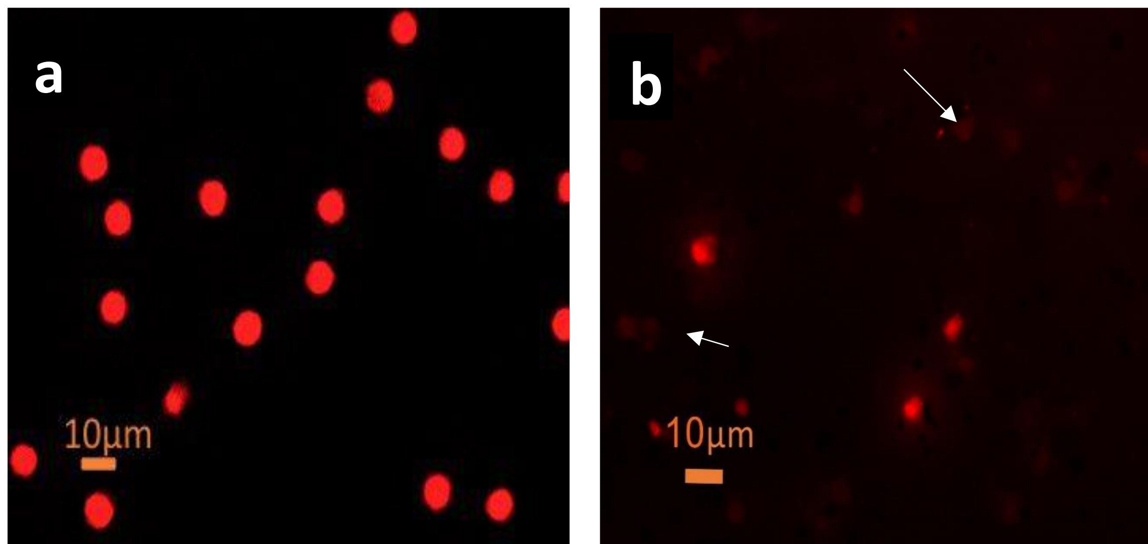


Figure 3.9 Vitality assay via cell staining using the LIVE/DEAD /Cytotoxicity Kit. THP-1 cell line exposed to *C. tamariscifolia*, *C. crinita* and *C. compressa* and incubated for 72h at 37°C. Treated cell lines and control stained with diluted Trypan blue stain and calculated with the Countess II FL Automated Cell Counter instrument equipped with standard Light Cubes. The histogram and the picture show the counts of live cells in the population, which fluoresce green, and dead cells, which fluoresce red.

3.2.7 *Cystoseira tamariscifolia* methanol extracts exhibit apoptosis and nuclei fragmentation on HL60 and THP-1 cell lines

To explore the cytotoxic effect of *C. tamariscifolia* methanol extracts and whether the extracts induced cell death via apoptosis and nuclei fragmentation, fluorescent staining of cancer cells nuclei by propidium iodide was conducted and observed using a fluorescent microscope. Cancer cells (A) HL60 and (B) THP-1 were treated with methanol extracts at the concentration of 150 µg mL^{-1} for 48h. As shown in figure 3.10, fragmented nuclei and apoptotic bodies were seen in the *C. tamariscifolia* extracts-treated cells (b), but not in the control treatment (a). Experiments were carried out in three replicates, and this result is in good accordance with that of the cell vitality assays.

(A)



(B)

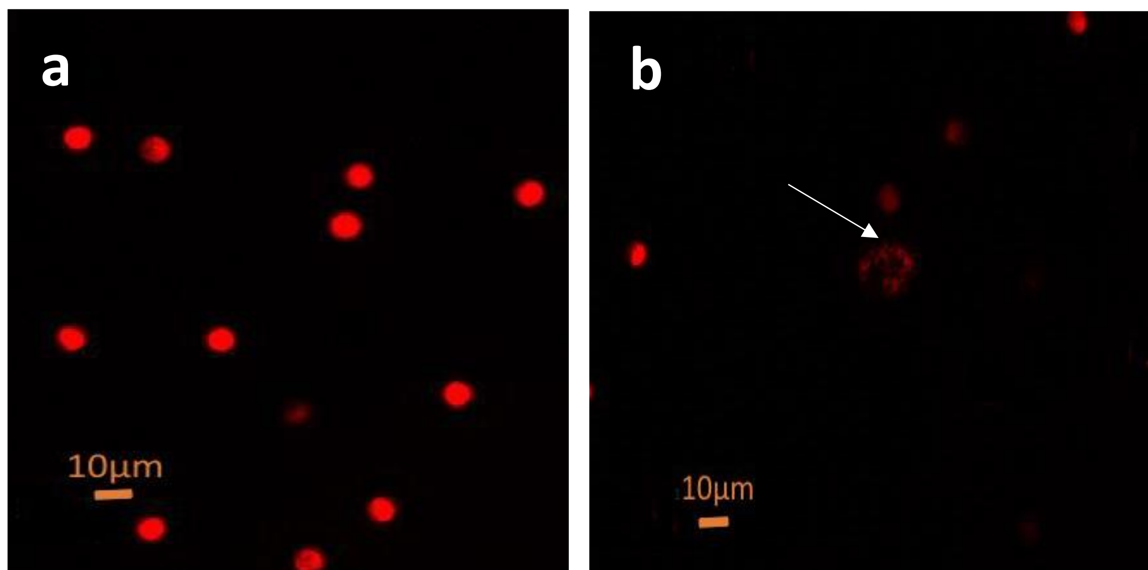


Figure 3.10 Fluorescent staining of cancer cells nuclei by propidium iodide. Cancer cells (A) HL60 and (B) THP-1 were treated with %100 methanol extracts at the concentration of 150 µg mL^{-1} for 48h. Fragmented nuclei and apoptotic bodies were seen in the *C. tamariscifolia* extracts-treated cells (b) but not in the control treatment (a). Magnification 200×

3.3 **Discussion**

Cystoseira genus is a rich source of natural products which are known as promising sources of anticancer metabolites. In this chapter, we aim to expand the knowledge of the effect of the extraction method and seasonality on the biochemical composition and the cytotoxic activity of the *Cystoseira* genus, which could lead to a novel drug for the pharmaceutical industry. The proximate biochemical composition and *in vitro* cytotoxic activity were determined in three *Cystoseira* species. *C. crinita* and *C. compressa* were collected from Libya while *C. tamariscifolia* was gathered from the U.K. coast in four seasons in order to study the seasonality of the biochemical composition and the cytotoxic activity in *C. tamariscifolia*.

The biochemical composition of *Cystoseira* macroalga has been previously studied by various authors (Duffy & Hay, 1990; Vizetto-Duarte *et al.,* 2016; Hentati *et al.,* 2018; Celenk & Sukatar, 2020). In line with these studies, *C. tamariscifolia C. crinita* and *C. compressa* studied in this thesis have certainly presented a high level of polyphenols contents, especially in *C. tamariscifolia* and *C. crinita*. Concentrations of polyphenols and flavonoids were high, a result in accordance with previous research showing brown seaweeds to be good sources of polyphenolic and flavonolic compounds (Thomas & Kim, 2011; Alghazeer *et al.,* 2016). At the same time, the polysaccharide has shown low concentration in *Cystoseira* species compared with the earlier studies. This could be referred to the fact that the extraction methods followed in our study are more designed to extract polyphenols sufficiently. Methanol (100%) was generally the most effective solvent for extracting all compounds, although all solvents gave detectable levels of all the investigated metabolites, the exception being chloroform that interfered with the BCA assay for proteins. A similar pattern of results was obtained by Mhadhebi *et al.* (2011) and Yegdaneh *et al.* (2016), who also concluded that methanol and chloroform extractions contain high chemical composition, especially polyphenols. This result can be explained by the difference in secondary metabolite polarity. For example, the high variation in the structures and both hydrophilic and hydrophobic parts of polyphenols (Li *et al.,* 2011) allow them to extract typically in polar solvents including methanol and water, but some can also extract in low polarity solvents such as chloroform (Airanthi *et al.,* 2011; Vizetto-Duarte *et al.,* 2016), which may explain the high levels of polyphenols in chloroform extracts of *Cystoseira*. Protein represented the least common of the four biomolecules.

The result of the cytotoxic activity assessments of *C. tamariscifolia, C. crinita* and *C. compressa* extracts, in general, indicate that methanol and chloroform extract of *C. tamariscifolia* and *C.*

crinita have potent inhibitory activity of cell vitality against HL60, THP-1 and REC-1 cell lines with the same comparable type of *C. compressa* extracts. The findings are in line with previous results by Duarte (2016), who demonstrated that a hexane extract of *C. tamariscifolia* had a high level of cytotoxic activity against AGS, HCT-15 and HepG2 human cell line with IC_{50} values of 32.36, 23.59 and 13.15 µg mL^{-1} respectively. However, the effect of our extracts on HL-60 and REC-1 cells was considerably more potent. Despite the fact that *C. crinita* extracts are considered one of the most potent anticancer agents used in several studies against various kinds of cancer cell lines (Mhadhebi *et al.,* 2011), diverse success degrees are found in our research for the treatment of different types of cancer with *C. crinita* extracts. Our results also showed that *C. compressa* generally had the lowest cytotoxic activity against all cancer cell lines, which is similar to a result reported by Kosanić *et al.* (2015) and Güner *et al.* (2015). These disparate outcomes of cytotoxicity activity between extracts and cancer cell lines were most likely influenced by the environmental variability of *Cystoseira* species, the nature of the active ingredients, the differences between cancer cell lines and the used extraction solvents. The evaluation of the cytotoxic activity of *Cystoseira* species was conducted following the protocol of the American Cancer Institute (NCI), which recommends that IC_{50} values up to 30 µg mL^{-1} of the crude extracts should be considered significant when the MTT method is used (Geran *et al.,* 1972). This being the case for the methanol and chloroform extracts of *C. tamariscifolia*, against both HL60 and REC-1 with IC_{50} values of 2.32 ± 0.21, 7.92 ± 0.12 5.10 ± 0.36 and 6.04 ± 0.9 µg mL^{-1}, respectively. In fact, the very low IC_{50} values of the crude extracts suggest that the effective cytotoxic ingredients in the crude extract could be at a much lower concentration which may make them very valuable anticancer agents. HL-60 and REC-1 cells showed the greatest susceptibility to *C. tamariscifolia* compared to PC3, whereas the cytotoxic potency of the extracts is between approximately 17 and 62 fold higher than the corresponding effects on PC3 cells. The variation of the cytotoxicity effect among the cancer cell lines might be attributed to the fact that cancer cells possess differences in their genetic makeup, morphology and doubling time, resulting in differential susceptibility to the same cytotoxic agent (Hanahan & Weinberg, 2011). For instance, in this study, prostate cancer cell line PC3 recorded the occurrence of resistance against the extracts in most cases compared to other cells lines. The resistance could attribute to their function as they produce large amounts of nutrients for supplying sperm with the source of energy necessary for their vitality and motility, which could reflect in the prostate high cell vitality (Caiazza *et al.,* 2019). The overexpression of the drug resistance gene (MDR-1)-encoded P-glycoprotein could also be identified as a cancer drug-resistant mechanism in the PC3 cells (Li *et al.,* 2011).

The MTT assay has been widely used to assess cell vitality and cytotoxicity by measuring the cellular metabolic activity of cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes such as mitochondrial succinate dehydrogenase, which reduces MTT to catalyzed MTT-formazan and therefore can be measured (Chacon *et al*., 1997). However, cytotoxicity assays usually require staining and microscopic evaluation to observe the morphology of cells as well as counting live and dead cells. The trypan blue dye exclusion test is commonly used for counting the number of viable and dead cells. The principle of the trypan blue dye test is that the trypan blue diazo dye is negatively charged and will not interact with cells unless its membrane is damaged, so only dead cells would be stained (Chan *et al*. 2020). In this study, the potent cytotoxic activity of the *Cystoseira* methanol extracts was confirmed by trypan blue staining data. The methanol extracts of *C. tamariscifolia, C. crinita* and *C. compressa* significantly decreased cell vitality in both HL60 THP-1 cell lines by up to 96%. The cytotoxicity activity against the two cell lines was slightly higher than it was found when the MTT assay was used, especially in the case of *C.compressa*. This could be explained by trypan blue ability to change the morphology of the cells, which could lead to cytotoxicity over-estimation (Chan *et al*. 2020). HL60 and THP-1 cell lines treated with *C. tamariscifolia* extracts were also observed using propidium iodide staining. They presented typical morphological characteristics of apoptosis, including nuclear fragmentation, which could be evidence of the possible cytotoxicity mechanism of the bioactive compounds in the extracts.

In temperate seas, one would expect that seasonal differences would have an enormous influence on the concentrations of likely bioactive compounds and thus the bioactivity of those extracts. Surprisingly, little work has been performed on these links. Here we have investigated the effects of season and extraction method on primary/secondary metabolite concentrations in *Cystoseira tamariscifolia* extracts and their cytotoxic activity against three cancer cell lines. As might be expected due to the correlation with abiotic and biotic parameters, there was a clear seasonality to the levels of the metabolites, although this varied between compound and extraction method (Celis-Plá *et al.,* 2016; Rickert *et al.,* 2016; Cikoš *et al.,* 2018). As *C. tamariscifolia* begins to grow in the late winter with the most growth in the spring and summer before stopping in autumn, both the spring and summer polyphenol samples contained the highest levels of compounds for each extraction method except for chloroform extracts from winter. This is consistent with observations by Abdala-Díaz *et al.* (2006), who showed that polyphenol concentration in the tissue of *C. tamariscifolia* (in Spain from June 1988 to July 2000) ranged between 2% in the winter to 8% in summer. Polyphenols from species of the brown algal orders Dictyotales, Fucales and Laminariales, have been correlated with the tissue age and stage of the life cycle where they

showed significantly lower levels in new branches in late winter than older and bigger branches in summer (Denton *et al.,* 1990; Mannino *et al.,* 2014). For flavonoids, polysaccharides and protein, the picture was more complex with winter and autumn often offering at least one higher value than in spring/autumn. Given the temperate nature of the collection site, Autumn and Winter provide lower water temperatures, fewer hours of sunlight and increased wave action that will lead to decreased growth and investment into primary and secondary metabolites (Fleurence & Levine, 2016). This may also coincide with reduced pressures of herbivory during these seasons and less requirement for secondary metabolites (Duffy & Hay, 1990). We found that the new growth of *C. tamariscifolia* was already clearly apparent in early March (Winter) with many fresh new phosphorescent fronds present presumably ready for the increased sunlight hours of spring and summer. Conversely, in October (Autumn), most of the thalli were showing early signs of senescence where the central axis and the primary laterals had elongated while the branches were relatively short and often covered in epiphytes. Despite this, autumn algae still contained high levels of some metabolites although polyphenol levels were generally lowest in autumn, but this depended on the extraction method. One explanation of this result could be attributed to the increase in the need for the secondary metabolites as a protection mechanism against living organisms in summer more than winter; for example, herbivores are often active primarily during the summer (Duffy & Hay, 1990; Jormalainen & Honkanen, 2008). Another reason for the increase of the secondary metabolites in summer may be due to the photoprotective role against the high radiation dosages in summer daylight (Connan *et al.,* 2004). They also contribute to the protection from oxidative stress, bacterial infection and epiphytes and perform a role in algal reproduction, which all increase in summer (Plouguerné *et al.,* 2006; Ferreres *et al.,* 2012; Jennings & Steinberg, 1997; Thomas & Kim, 2011).

Our results demonstrate that there is substantial variation not only in the levels of primary and secondary metabolites according to season and extraction method but that there is also significant variation in their cytotoxic effects. In particular, our results clearly underline the need to test extracts from different seasons and use different solvent extraction protocols. Of particular interest, our data suggest that generalisations cannot be drawn with respect to season or extraction method and that there is no best season or extraction method when results are compared between cell types. Indeed, we carried out statistical analyses to examine any potential interactions between metabolite composition and the cytotoxicity of the extracts with season and extraction method. There was no clear interaction between seasons and extraction method could affect the cytotoxicity of C. *tamariscifolia* extracts on cancer cell lines. It would be tempting to assume that

summer (or an early or midpoint of the growing season) might be the best time to collect material, but our results show that this is not necessarily the case in all cell lines or extracts. It is likely that different compounds are responsible for the observed effects. For example, the difference between the impact of the summer extracts in organic solvents of *C. tamariscifolia* for HL-60 cells and the aqueous winter extracts on PC3 cells highlights this difference. We believe that it is possible that many promising extracts potentially containing novel cytotoxic/chemotherapeutic agents may have been discarded in the past where samples were taken in a single season or from one region, extracted using a single solvent or tested using a single cell line.

We suggest that, where possible, samples are taken at multiple times of the year that represent different phases in the growth or annual life cycle of the plant/species in question. Given that many of the bioactive compounds with potential use medically are probably produced in response to some type of biotic or abiotic stress, it may also be good practice to include the presence of any apparent stressors (seasonal temperature, irradiance, herbivores, for example) into any consideration of sampling times to maximise the possibility of finding novel compounds.

Given the abundance of bioactive compounds present in brown alga *Cystoseira,* which already proved to have cytotoxic activity, we assumed that those bioactive compounds have more bioactivities than have been described here. It was interesting to continue investigating *Cystoseira* extracts for anticancer activity via immunomodulation as we aimed to test our *Cystoseira* extracts for stimulating the immune cells to fight cancer. However, surprisingly the extracts showed very interesting results on the inhibition of cytokine release. We then decided next to investigate the anti-inflammation activity of *Cystoseira* spp. extracts.

CHAPTER 4

METHANOL EXTRACT OF BROWN SEAWEEDS *CYSTOSEIRA* SPP. INHIBITS PRO-INFLAMMATORY RESPONSE IN STIMULATED HUMAN MACROPHAGE-LIKE CELLS

4.1 Introduction

The inflammatory response is a controlled multifactorial process that occurs as a defence mechanism against infections or to promote tissue repair. Macrophage plays critical roles in this immune reaction, allergy and inflammation. (Idriss & Naismith, 2000; Mills *et al.,* 2000). Macrophage activation by endotoxins such as lipopolysaccharides (LPS) results in initiating and maintaining specific immune responses by releasing different types of inflammatory mediators including cytokines such as tumour necrosis factor-alpha (TNF-α) and Interleukin-beta (IL-1β) (Cheong *et al.,* 2016; Madrigal *et al.,* 2014). Over-expression of the inflammatory mediators in macrophage is involved in many inflammation related diseases, such as sepsis, atherosclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease, autoimmune diabetes, arthritis, inflammatory bowel disease, asthma, and chronic obstructive lung disease and cancer (Eisenman *et al.,* 2017; Lee *et al.,* 2013; Qiang *et al.,* 2018, Frank *et al.,* 2019; Allavena & Mantovani, 2012). The dysregulation of the inflammatory response plays a large role in the development of chronic diseases that result in severe tissue/organ damage and aberrant repair/remodelling. Immune cells become dysregulated and lose their self-limiting character as a result of persistent inflammation. For instance, in Rheumatoid arthritis synovial fibroblasts produce chemokines in response to IL-1 and TNF-α which bind to their receptors and attract inflammatory cells to the site of inflammation. Normal T expressed and secreted (RANTES)/CC ligand 5 (CCL5), a powerful CC chemokine is proven to play a significant role in Rheumatoid arthritis pathogenesis, is one of these chemokines that is controlled on the activation. (Mor *et al*., 2005; Patel *et al*., 2001). Tissue production of the pro-inflammatory cytokines IL-1β and TNF-α correlates with the intensity of the systemic inflammatory response and with corticosteroid requirements in giant-cell arteritis (Hernandez-Rodriguez *et al*., 2004). Following a stroke, patients with giant-cell arteritis have higher levels of IL-1 and TNF, which promote antigen-specific (neuron or oligodendrocyte) Th1-cell or Th2-cell development. Neuron and oligodendrocyte damage, cerebral haemorrhage, EPVS, expanded perivascular space, microbleeds, and cerebral small vessel disease are all caused by the cellular immune response or humoral immune response. (Fu & Yan, 2018). Moreover, proinflammatory cytokines, are linked to atherosclerosis, arteriosclerosis, endothelial dysfunction, smooth muscle cell migration, vascular calcification, increased activity of metalloproteinases, extracellular matrix degradation, oxidative stress, elastolysis, and collagen degradation, which plays a major role in the stiffening of large arteries. The presence of arterial stiffness

indicates a higher risk of cardiovascular events. (Ayhan *et al*., 2015; Mozos *et al*., 2017). Also, Obesity-induced inflammation is characterized by the infiltration and retention of immune cells within the adipose tissue and the chronic release of pro-inflammatory cytokines, including TNF-α, IL-1β, IFN-γ, and IL-6 (Gerriets & MacIver, 2014). This obesity-induced, low-grade systemic inflammation has been linked to insulin resistance, diabetes, arterial stiffness, endothelial dysfunction, and increased blood-brain barrier permeability (Frasca *et al*., 2017). Ageing by itself is characterized by a state of chronic inflammation, known as inflammation, and obesity superimposed on ageing represents an additional risk factor for chronic disease and age-related complications (Ahnstedt *et al*., 2018). Moreover, the activity of the TNF-α pathway was associated with the risk score of microalbuminuria in patients with type 1 diabetes (Purohit *et al*., 2018). Thus, inhibition of the production of these inflammatory mediators is an important target in the treatment of inflammatory diseases. Historically, immune diseases treatment includes synthetic non-steroidal drugs, corticosteroids, synthetic disease-modifying anti-inflammatories and immunosuppressants. However, those drugs are usually associated with adverse reactions, as gastrointestinal problems, ranging from mild to severe dyspeptic symptoms, the development of gastric or duodenal ulceration, haemorrhage or perforation. They may be also associated with cardiovascular, liver, kidneys complications, opportunistic infections, and other events which may lead to hospitalisation or death (Russell, 2001; Rainsford, 2007; McCarberg & Gibofsky, 2012). Therefore, new alternative anti-inflammatory drugs with lesser side effects and low toxicity based on their effects as anti-cytokine agents are needed. These drugs are now being heralded as the new therapies to control those diseases where cytokines of chronic inflammatory and neurodegenerative diseases are manifest.

The brown seaweed *Cystoseira* is exposed to light and high oxygen concentrations that induce the formation of inflammatory mediators. Thus, it is able to generate the necessary compounds to protect itself from external factors such as pollution, stress and UV radiation. Therefore, *Cystoseira* can be considered as a potential anti-inflammatory source. *Cystoseira* is distributed widely abundantly on many Mediterranean countries' coasts and is well recognised to be a rich source of bioactive components such as fatty acids, polysaccharides, polyphenols, flavonoids, and peptides, which have been proposed as immune functions modulation agents (Liu *et al.,* 1997; Zhang *et al.,* 2015; Yang & Zhang, 2017). Among the biochemical compounds of *Cystoseira* for the treatment of immune diseases, fatty acids are related to present anti-inflammatory activity and act as physiological and metabolic regulators with a low grade of toxicty (De La Fuente *et al*.,2021; Mahmoud *et al*., 2021). *Cystoseira* has relatively a high lipids content in which polyunsaturated fatty acids such as linoleic acid and arachidonic acid

are abundant (Belattmania *et al*., (2018). However, very few studies have been conducted on the anti- inflammatory activity of the fatty acids from *Cystoseira.* Recently, a study by De La Fuente *et al* (2021) demonstrated that a strong anti-inflammatory effect of *C. amentacea* extracts was able to abate intracellular ROS production in H2O2 -stimulated macrophages and fibroblasts and to strongly inhibit LPS-induced inflammatory mediators, such as NO production and IL-1α, IL-6, cyclooxygenase-2 and inducible NO synthase gene expression in RAW 264.7 macrophages. Fatty acids anti-inflammatory mood of action could also be via increasing the mRNA expression of IL-10 and TGFβ and reducing the expression of TNF, IFN-γ, IL-1β, IL-6, and IL-17A in the blood and skin lesion. (Choudhary *et al*., 2021). Polysaccharides are also abundant in *Cystoseira* and were found to stimulate human lymphocytes proliferation and to stimulate monocyte to produce TNF-α (Shan *et al.,* 1999). They also showed an immune modulation on the M2 subtype through affecting lymphocytes with low side effects (Sun *et al.,* 2016; Atashrazm *et al.,* 2015). For instance, fucoidan is reported to act on different stages of the inflammatory process via reducing the expression of genes of COX-2, IL-1β, and the NF-κB signalling pathway (Apostolova *et al*., 2020). Polyphenols dependently reduce the pro-inflammatory activation of M1 macrophage-like cells with a significant reduction in the secretion rate of cytokines when stimulated by LPS. Polyphenols also increased cytokine IL-10 secretion, which promotes the anti-inflammatory M2 macrophage differentiation (Yahfoufi *et al.,* 2018; Aharoni *et al.,* 2015). The mechanism that explains how extracts enter into the cells and the involved molecular pathways is still, to some extent, unclear. However, recently it has been revealed that polyphenols influence macrophage phenotype anti-inflammatory state, interrupt cell signalling and gene expression of many cytokine genes (Ramiro *et al.,* 2005; Yahfoufi *et al.,* 2018).

Since methanol crude extracts from *C. tamariscifolia*, *C. crinita* and *C. compressa* as an active ingredient are more popular over the pure pharmacological compounds and have has not been investigated extensively for their anti-inflammatory properties (Custódio *et al.,* 2016; Mhadhebi *et al.,* 2011). The objective of the current study was to evaluate *Cystoseira* anti-inflammatory effect in LPS, LTA, heat-killed and live *Escherichia coli* stimulated M1 macrophages-like cells based on the results of using the enzyme-linked immunosorbent assay (ELISA) assay. Also, to explore whether the *Cystoseira* extracts contain endotoxins that could interact with and block the LPS used for stimulating M1macrophage-like cells, a Limulus Amebocyte Lysate (LAL) endotoxin detection assay was conducted. The cytotoxic activity of the extracts on the macrophage-like cells was measured using MTT assay and the disc diffusion

method was performed to investigate the antibacterial activity of the extracts against Gram-positive and Gram-negative bacteria. Our results showed that *C. tamariscifolia* and *C. crinita* methanol extracts had a significant anti-proinflammatory activity on activated M1macrophage-like cells. On the other hand, all extracts did not exhibit any antibacterial activity on tested microorganisms. Collectively, our findings provided a clear insight into the wide range of impact by which *Cystoseira* methanol extracts inhibited the inflammation by suppressing the release of pro-inflammatory cytokines in M1 macrophages-like cells. Therefore, this study might be useful for chemists to develop anti-proinflammatory functional material from *C. tamariscifolia* and *C. crinita* in the future.

4.2 Results

4.2.1 *Cystoseira* extracts suppress pro-inflammatory cytokines TNF-α and IL-1β release in human LPS-stimulated macrophage-like cells

We were interested in investigating whether *C. tamariscifolia*, *C. crinita* and *C. compressa* suppress the release of TNF-α and IL-1β in LPS-stimulated macrophage-like cells. M1 macrophage-like cells were stimulated for two hours by LPS and then were incubated for 18 hours with methanol extracts of the *Cystoseira* species. The results as shown in figure (4.1), indicate that methanol extract of *C. tamariscifolia* significantly inhibited the release of TNF-α and IL-1β to approximately 20% to 50% respectively at an extract concentration of 250 $\mu g\ mL^{-1}$ in comparison with the LPS controls. *C. crinita* reduced the release of TNF-α and IL-1β to 40% and 50%, respectively when compared to positive controls. At the same time, *C. compressa* had the lowest anti-proinflammatory activity on the IL-1β and no notable reduction in TNF-α release. It is worth mentioning that the experimental setup included control to be tested which is the methanol that we used to extract the bioactive compounds. We found that the methanol had no influence on the release of cytokines from LPS-stimulated M1macrophage-like cells in any of the experiments.

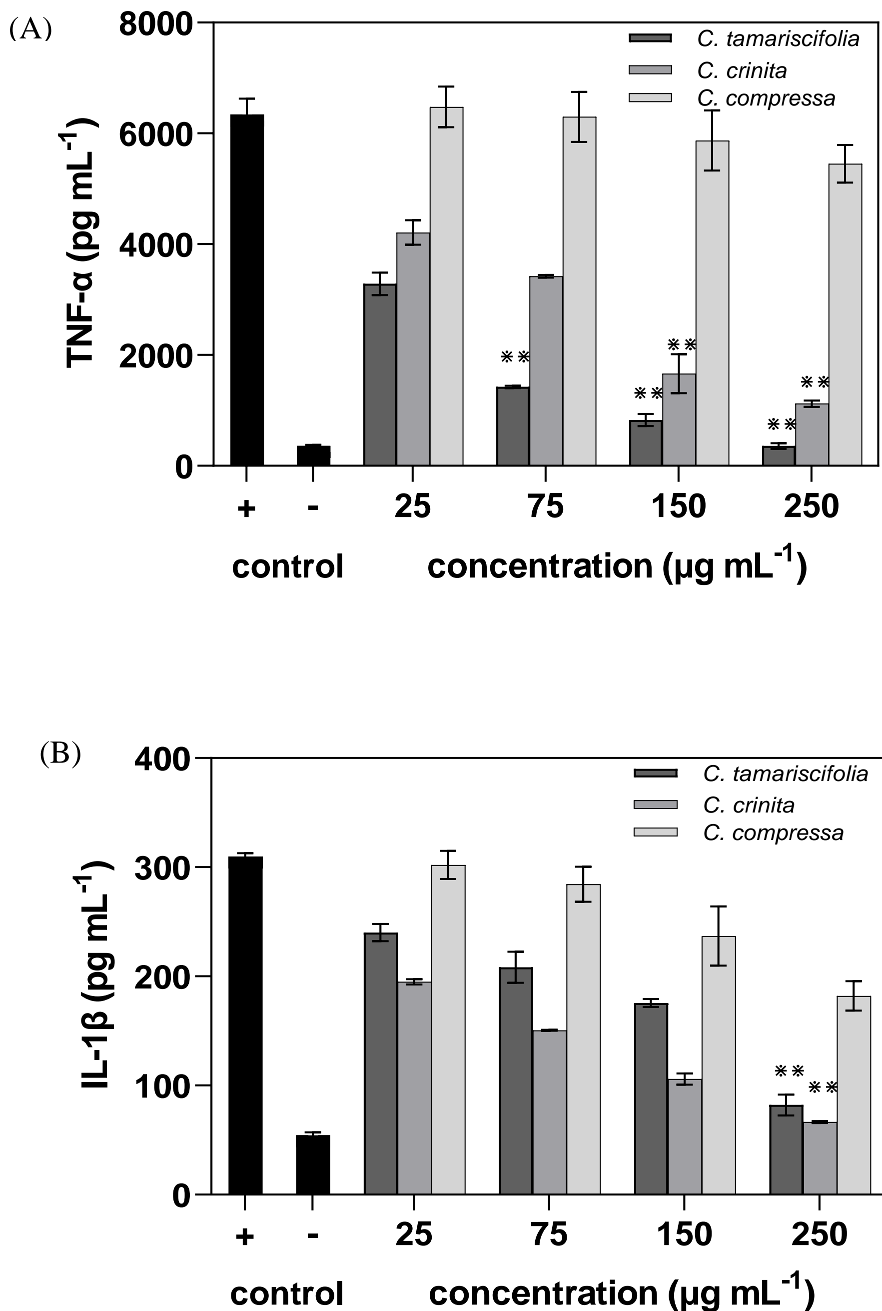


Figure 4.1 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in LPS-stimulated macrophage-like cells for 20 h incubation. After 20 h incubation, TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and LPS (1 µg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** $P<0.05$ vs LPS alone.

4.2.2 Effects of *Cystoseira* methanol extracts on lipoteichoic acid (LTA) induced TNF-α release

To examine the ability of the methanol extracts of *Cystoseira* on the inhibition of LTA induced TNF-α release from M1 macrophage-like cells and also for better understanding the anti-proinflammatory mechanism of the extracts, methanol extracts were added to the media and LTA-stimulated M1macrophage-like cells in concentration dependent manner. The results presented in Figure (4.2) show that *C. tamariscifolia, C. crinita* and *C. compressa* significantly suppressed the release of TNF-α from LTA-stimulated M1macrophage-like cells. Among the extracts, the result shows that the *C. tamariscifolia* extract had a substantially higher TNF-α release inhibitory effect than *C. crinita* and *C. compressa*.

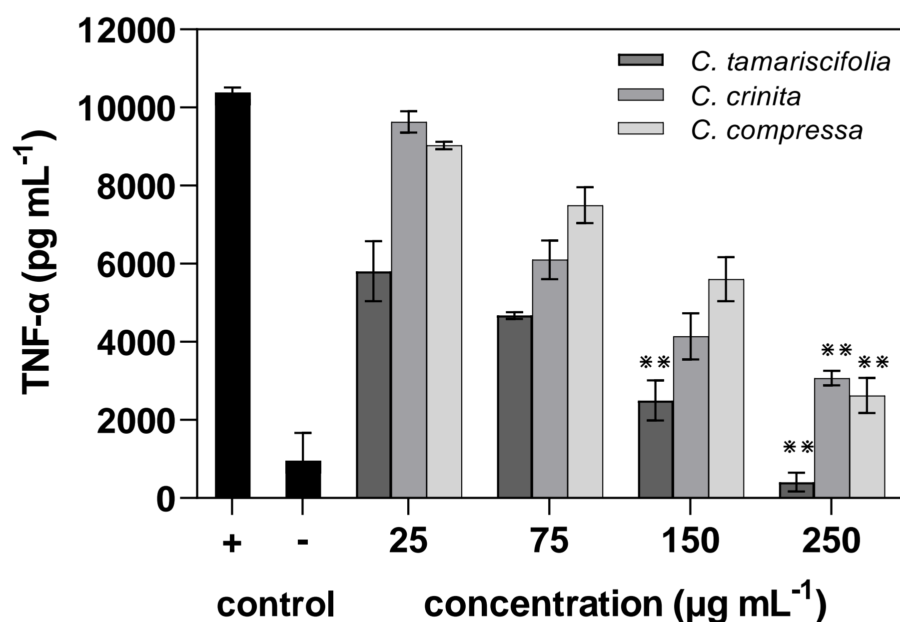


Figure 4.2 Effect of *Cystoseira* extracts on TNF-α release in LTA-stimulated macrophage-like cells. After 20 h incubation, TNF-α released into the culture medium were assayed by the ELISA method. No treatment control (-) and LTA alone (5 μg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** $P<0.05$ vs LTA alone.

4.2.3 The cytotoxic effect of the methanol extracts of *Cystoseira* species on M1 macrophage-Like cells

Considering that the inhibitory effect of methanol extracts of *Cystoseira* species on pro-inflammatory cytokines release from macrophages may be due to several possibilities and mechanisms, we decided to investigate how the extracts of *C. tamariscifolia*, *C. crinita and C. compressa* suppressed the release of pro-inflammatory cytokines TNF-α and IL-1β in stimulated macrophage-like cells. We decided next to begin by assessing whether the extracts reduced the release of cytokines by killing the macrophage-like cell. M1macrophage-like cells were treated by the *Cystoseira* spp methanol extracts with increasing concentration overnight, and the cytotoxic effect was assessed by using MTT assay and the data shown in figure (4.3). After 20 hours of incubation, *C. tamariscifolia*, *C. crinita* and *C. compressa* did not show a cytotoxic effect on M1 Macrophage-like cells.

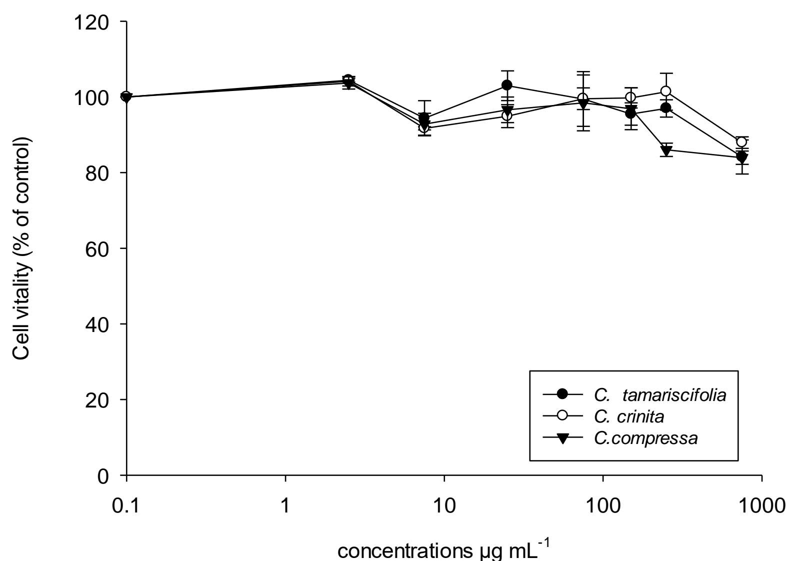


Figure 4.3 Percentage of cell vitality in comparison to control (mean ± SD; n=3). M1 Macrophage-like cells were exposed for 20 h to 100% methanol crude extracts of *Cystoseira* species.

4.2.4 Methanol extracts of *Cystoseira* spp exhibit anti-endotoxin activity

To explore the anti-endotoxin activity of C. *tamariscifolia* and *C. crinita* methanol extracts and whether the *Cystoseira* extracts contain endotoxins that could interact with and block the LPS used for stimulating M1macrophage-like cells, a Limulus Amebocyte Lysate (LAL) endotoxin detection assay was conducted, and the results are shown in figure (4.4). No endotoxins contamination was detected in *Cystoseira* extracts. Both *C. tamariscifolia* and *C. crinita* methanol extracts significantly blocked the endotoxin LPS at $P < 0.05$ but not complete blocking. Since extracts did not block endotoxin completely and for further exploration of how extracts suppressed the TNF-α and IL-β release, we, therefore, performed additional pre-treatment experiments. As we normally treat M1macrophage-like cells with extract after two hours to allow LPS to stimulate the release of the cytokines, here, we also added the extract with the LPS at the same time, and the results are shown in figure (4.5 A, B and 4.6 A, B). The methanol extracts of *C. tamariscifolia* and *C. crinita,* a concentration of 250 µg $mL^{-1,}$ significantly inhibited $P<0.05$ the release of both TNF-α and IL1-β from LPS-stimulated M1macrophage-like cells versus LPS control alone. More importantly, the result showed that there was a notable increase in the inhibition when the extract was added after two hours of stimulation in TNF-α. However, no noteworthy difference in the case of the inhibition of and IL-β release.

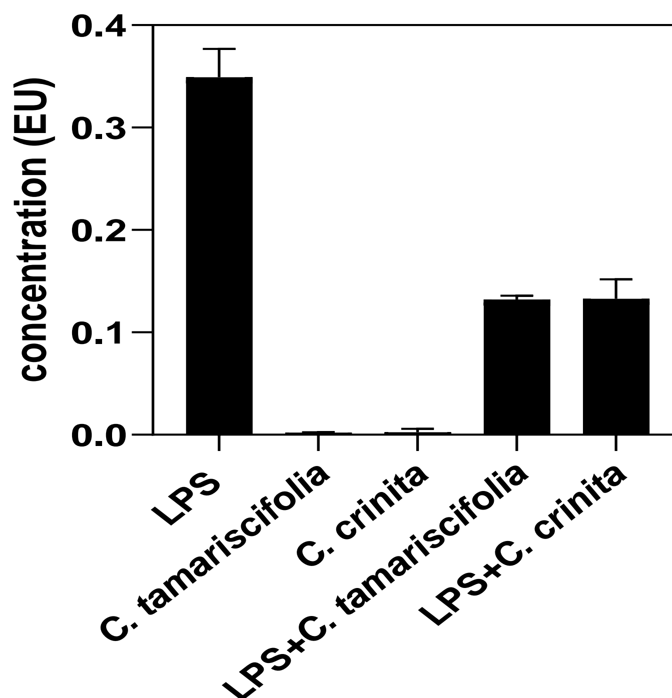
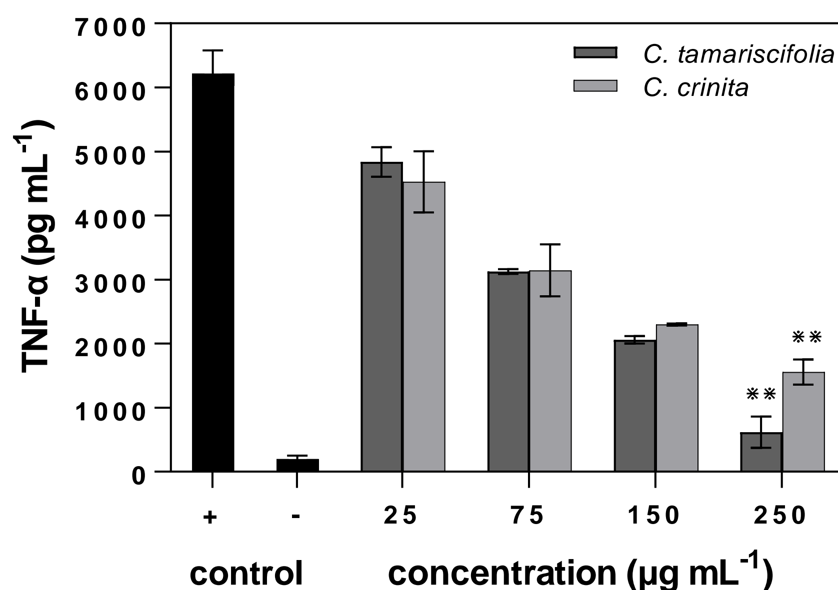


Figure 4.4 Endotoxin detection in extracts and LPS with extracts using the endpoint chromogenic LAL assay. LPS (1 µg mL^{-1}) and the mixture of LPS and extracts *C. tamariscifolia* and *C. crinita* (1 mg mL^{-1}) were incubated with LAL and substrate. After the incubation period, the absorbance of the substrate was measured. The graph shows that no endotoxin contamination in extracts.

***Cystoseira* extracts added together with LPS**



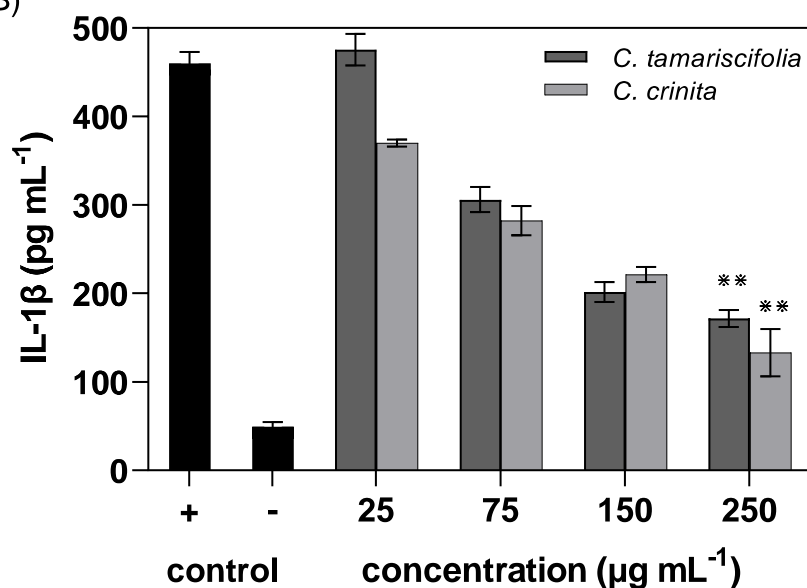


Figure 4.5 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in LPS-stimulated macrophage-like cells. *Cystoseira* extracts were added together with 1 μg mL^{-1} LPS. After 20 h incubation, TNF-α and IL-1β produced and released into the culture medium were assayed by the ELISA method. No treatment control (-) and LPS (1 μg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** $P<0.05$ vs LPS alone.

***Cystoseira* extracts added after LPS**

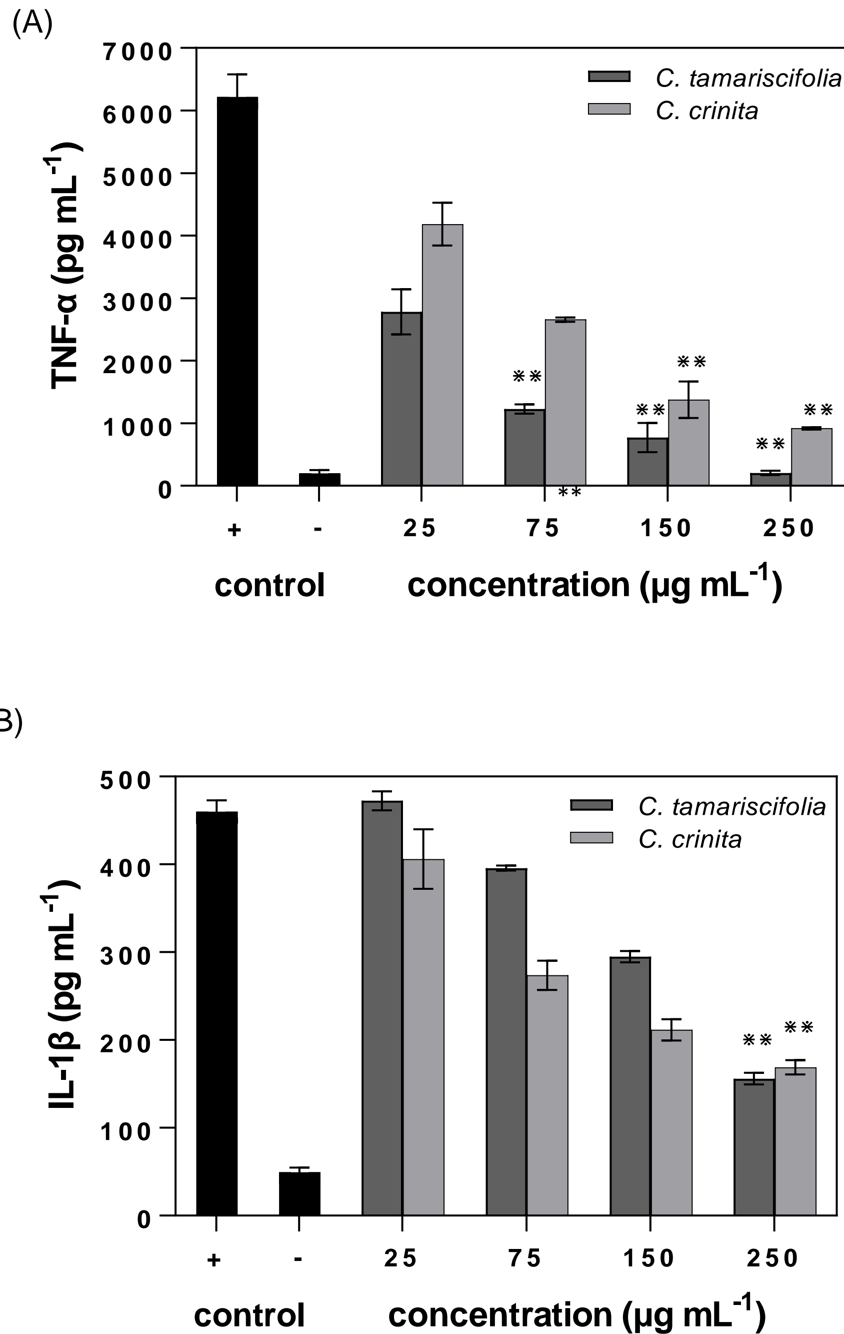


Figure 4.6 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in LPS-stimulated macrophage-like cells. *Cystoseira* extracts were added after 2 h of adding 1 µg mL^{-1} LPS. After 20 h incubation, TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and LPS (1 µg mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. * P<0.05 vs LPS alone.

4.2.5 Effects of *Cystoseira* methanol extract on heat-killed and live *Escherichia coli* induced TNF-α and IL-1β release

In addition to LPS and LTA, bacteria produce several other types of endotoxins that can stimulate immune cells responsible for releasing inflammatory cytokines into the human body. Since *C. tamariscifolia* and *C. crinita* had a significant inhibition on the release of cytokines from LPS and LTA-stimulated M1macrophage-like cells, we were interested in examining whether the extracts can also block other endotoxins or interrupt different potential pathways. Initially, an experiment was conducted to endeavour to ascertain the appropriate concentration of the bacteria that was equivalent to the stimulation effect of 1 μg mL^{-1} of LPS. The results showed that bacteria *E. coli* stimulated the release of TNF-α in a concentration dependent fashion figure (4.7). The result also showed that, activation by bacteria *E. coli* at a concentration of 10^9 cell mL^{-1} were similar to those obtained with the LPS at the concentration of 1 μg mL^{-1}. Therefore, we used this concentration of bacteria for all experiments as the best concentration for M1 macrophage-like cells stimulation.

The results of TNF-α and IL-1β, release in M1macrophage-like cells pre-treated with heat-killed and live *E. coli* and then *Cystoseira* methanol extracts are shown in Figures (4.8 A, B and 4.9 A, B). Exposure of M1macrophage-like cells to heat-killed and live *E. coli* at concentration 10^9 cell mL^{-1} stimulated both TNF-α and $IL^{-1}β$ release to the media at 20 h time compared with the negative control. *C. tamariscifolia* and *C. crinita* methanol extracts at (250 μg mL^{-1}) treatment had identical significant suppression ($P<0.05$) effect of approximately 85% and 60% on TNF-α and IL-1β release when M1macrophage-like cells were stimulated by heat-killed *E. coli* bacteria. This was not the case when M1macrophage-like cells were stimulated by live *E coli*, as there was a notable difference between the inhibition of TNF-α and IL-1β. While the anti-proinflammatory activity of *C. tamariscifolia* and *C. crinita* extracts at 250 μg mL^{-1} was significant ($P<0.05$) against the TNF-α release. On the release of IL-1β, both *C. tamariscifolia* and *C. crinita* methanol extracts had almost no activity.

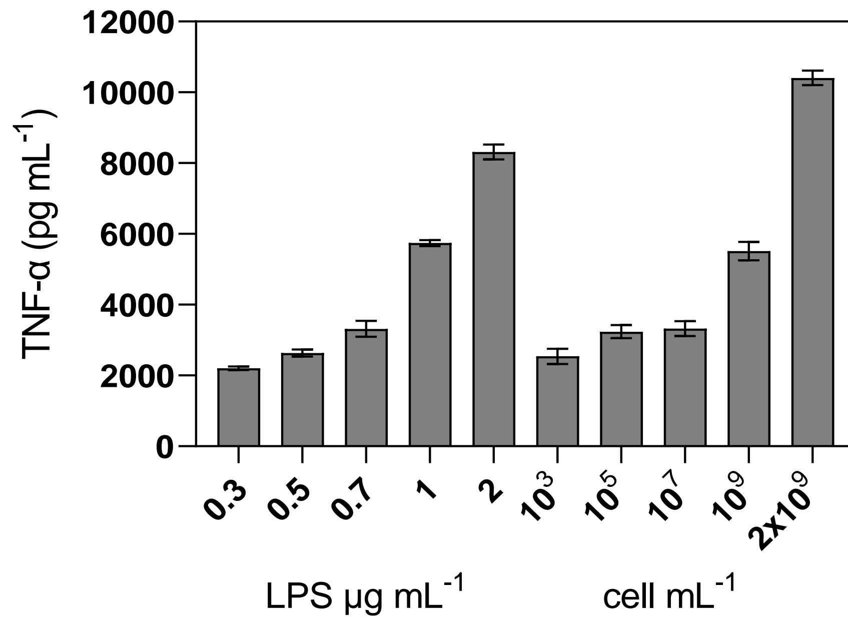


Figure 4.7 TNF-α release when M1 macrophage-like cells stimulated by heat-killed *E. coli* versus when stimulated by LPS. Cells were cultured for 20 h in the presence of increasing concentrations of both LPS and heat-killed *E. coli* bacteria. Data represent the mean value (mean ± SD) of the cytokine in pg mL^{-1} of cell culture supernatant for triplicate samples for each test treatment.

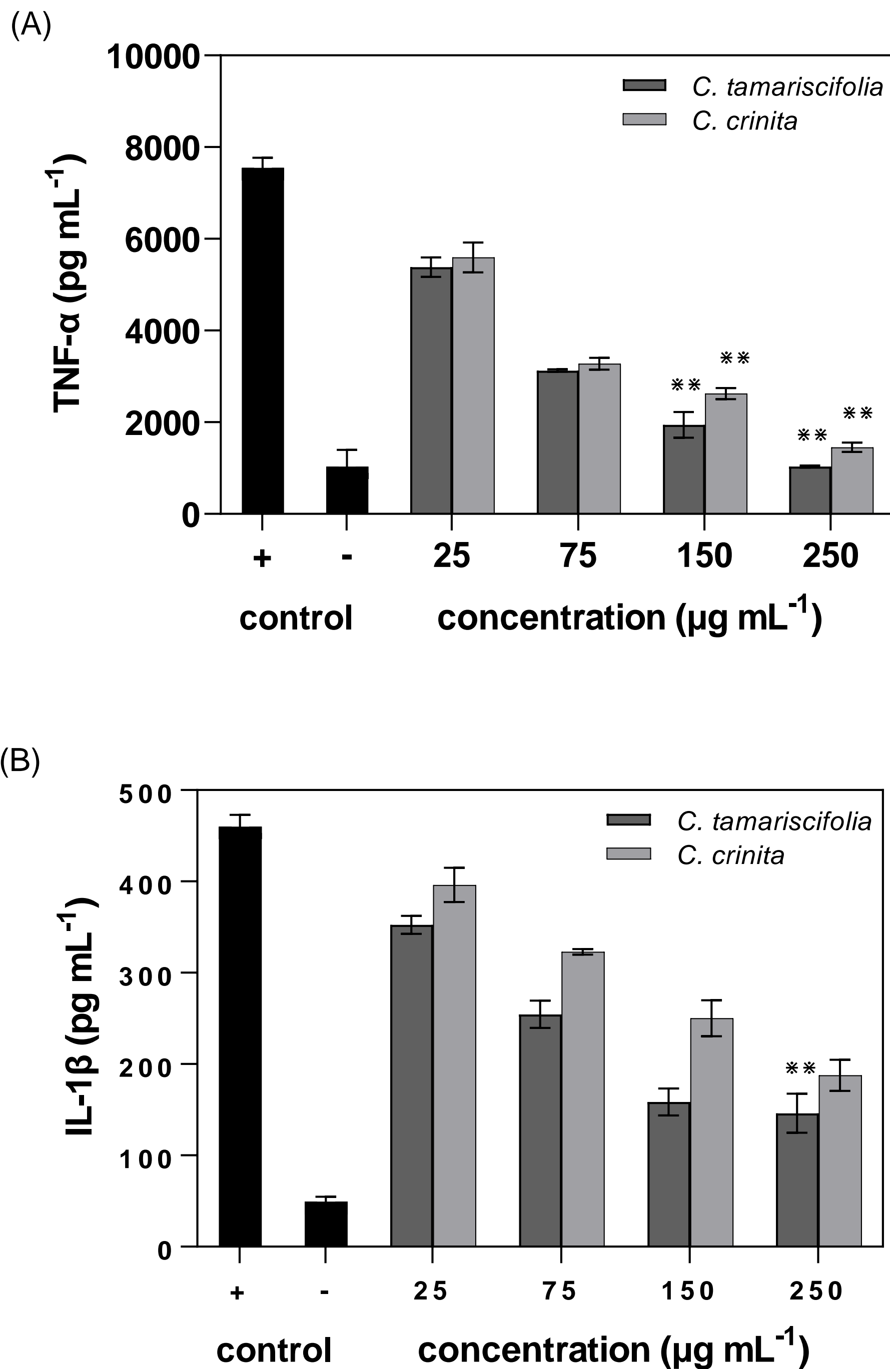


Figure 4.8 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in heat-killed *E. coli* k12-stimulated macrophage-like cells. After 20 h incubation, TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and heat-killed *E. coli* (10^9 cell mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** $P<0.05$ and vs heat-killed *E. coli* alone.

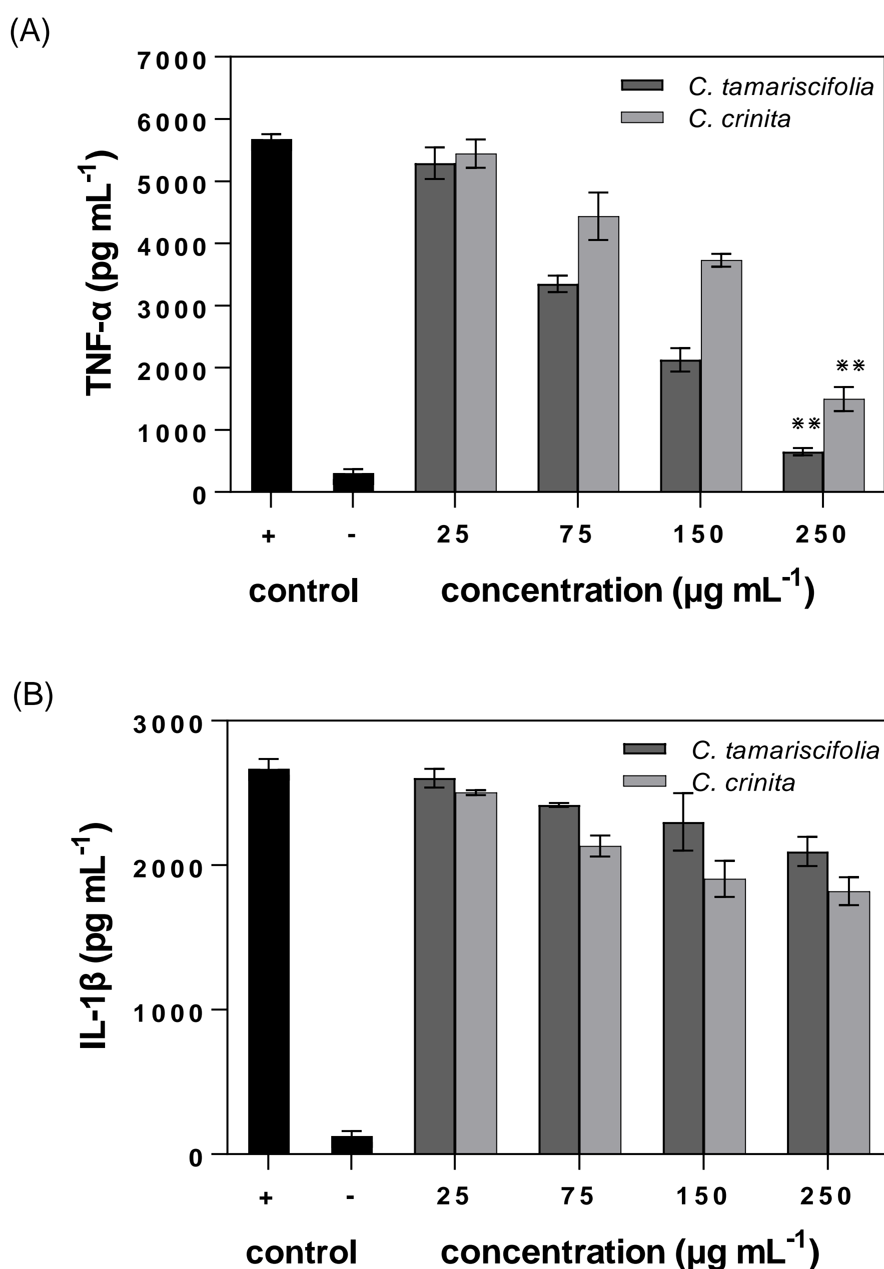


Figure 4.9 Effect of *Cystoseira* extracts on TNF-α (A) and IL-1β (B) release in live *E. coli* k12-stimulated macrophage-like cells after 20 h. TNF-α and IL-1β released into the culture medium were assayed by the ELISA method. No treatment control (-) and live *E. coli* (10^9 cell mL^{-1}) control (+) were also assayed. The data represent the mean ± SD of triplicate experiments. ** P<0.05 and vs Live *E. coli* alone.

4.2.6 The antibacterial activity of extracts of *Cystoseira* spp

Bacterial infection in the body may release endotoxins, stimulating macrophages to produce a higher level of pro-inflammatory cytokines. This could promote unwanted immune reactions that interfere with the anti-proinflammatory activity of *Cystoseira* extracts. We, therefore, tested the antibacterial activity of the *C. tamariscifolia, C. crinita* and *C. compressa* methanol extracts at (250 µg mL^{-1}) against Gram-positive *Staphylococcus aureus* Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* and using a disc diffusion method. As presented in table (4.1), the results indicate that all *Cystoseira* extracts did not exhibit antibacterial activity against tested Gram-positive and Gram-negative microorganisms when compared with the standard antibiotic gentamicin.

Table 4.1 The antibacterial activity of methanolic extracts of *Cystoseira* spp using disk diffusion assay. Average diameter (±SE) of inhibition zone of *C. tamariscifolia*, *C. crinita* and *C. compressa* methanol extracts at concentrations (1, 3, 6, 10 and 100 mg mL^{-1}) against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* strain K12 using disc diffusion method and gentamicin as a standard antibiotic.

Cystoseira spp	Dilution (mg mL^{-1})	Diameter of inhibition zone (mm)				
		Solvent	Gentamicin 10 µg mL^{-1}	*S. aureus*	*P. aeruginosa*	*E. coli*
C. tamariscifolia	1	-	17-20	-	-	-
	3	-	20-22	-	-	-
	6	-	21-23	-	-	-
	10	-	20-21	-	-	-
	100	-	22-23	1-3	-	-
C. crinita	1	-	20-21	-	-	-
	3	-	19-21	-	-	-
	6	-	17-18	-	-	-
	10	-	21	-	-	-
	100	-	22	-	-	-
C. compressa	1	-	19-20	-	-	-
	3	-	19-22	-	-	-
	6	-	19	-	-	-
	10	-	21-23	-	-	-
	100	-	20	-	-	-

4.3 Discussion

The *Cystoseira* genus is experiencing a growing interest in drug release among marine algae since it is moderately easy to obtain and a sustainable rich source of various bioactive composites with valued pharmaceutical potential to meet future therapeutic requirements such as anti-inflammatory drugs. This study evaluates the anti-proinflammatory properties of the methanol extract of *C. tamariscifolia, C. crinita* and *C. compressa* on the TNF-α and IL-1β release by activated macrophage-like cells. Pre-treatment experiments were performed *in vitro*, and the level of released cytokines was measured using ELISA, which is a rapid and reliable screening method broadly used for investigating the anti-inflammatory properties of samples.

Macrophages are essential members of the inflammation physiological defence mechanism that deregulated in association with several diseases which mediate the response of immune cells. They release pro-inflammatory mediators, such as TNF-α and IL-1β, which are important mediators of inflammation induced by macrophages during bacterial infections and tissue injury by increasing blood supply, enhancing vascular permeability and migration of immune cells to damaged sites (Genin *et al*., 2015). However, such an inflammatory response may cause tissue damage and can generate lethal consequences (Montalvão *et al*., 2016). Therefore, macrophages represent a good model for studying the anti-proinflammatory of *Cystoseira* extracts. However, owing to the difficulties of obtaining human monocytes and macrophages *in vitro* and also because of the metabolic and morphological similarity between THP-1 and the monocytes (Auwerx, 1991; Qin, 2012), we used human M1 macrophage-like cells differentiated from THP-1 by using Phorbol 12-myristate 13-acetate (PMA) as a model for the anti-inflammatory experiments. PMA is used commonly for differentiating THP-1 cell lines into macrophage-like cells (Foey & Crean, 2013; Genin *et al.,*2015). As a result of this differentiation, a number of changes appear on the differentiated cells such as gene transcription, an increase in the numbers of mitochondria and lysosomes, lack of division and an increase in phagocytosis and adhesion, changing in shape, development in Golgi apparatus and rough endoplasmic reticula (Kohro *et al.,*2004; Daigneault *et al.,* 2010; Verreck *et al.,* 2006; Sharif *et al.,* 2007; Tsuchiya *et al.,* 1982). Due to the malignant background of THP-1 cell lines, this model of M1macrophage-like cells may not display the whole response spectrum of M1 macrophages. However, M1macrophage-like cells have been largely proposed as a beneficial model for investigating macrophages in a number of studies (Auwerx, 1991; Foey & Crean, 2013; Qin, 2012). For instance, they have been used to study macrophages in the brain of Alzheimer's patients, cardiovascular system disease, type 2 diabetes and to study the

effect of the Mycobacterium tuberculosis replicates inside human macrophages (Meda *et al.,* 1995; Estrella *et al.,* 2011; Riek *et al.,* 2010). Collectively, M1 macrophages-like cells differentiated from THP-1 by using PMA still can be used to study the effect of the *Cystoseira* extracts on inhibiting the role of the M1 macrophage in promoting inflammation.

Bacterial cell wall components Lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria are essential immune stimulators of the innate immune system. The interaction between bacterial components and macrophages TLRs, which are essential proteins in the innate immune system, activates an immune signalling network, leading to the fast and temporal phosphorylation of a number of final signalling pathways (Koch *et al.,* 2014). The final result is the transcription of hundreds of inflammatory mediators that lead to broad inflammatory effects. However, growing evidence suggests a close link between inflammation and many chronic health conditions including diabetes, metabolic syndrome, cardiovascular disease, cancer, rheumatoid. Therefore, we first investigated the potential of anti-proinflammatory activity of the *Cystoseira* methanol extracts on LPS and LTA stimulated M1macrophage-like cells. Here we show that methanol extracts of C. *tamariscifolia* and *C. crinita* significantly inhibited the release of TNF-α and IL-1β from LPS or LTA-stimulated M1macrophage-like cells without cytotoxic effect. These findings are higher than the results in studies conducted by De los Reyes *et al.* (2013) and Zbakh *et al.* (2020). However, *C. compressa* did not show a notable anti-inflammatory effect which could be explained by the lower biochemical composition compared to the other two species. The inhibition of TNF-α and IL-1β release from stimulated M1 macrophage-like cells when treated with natural products may happen through several mechanisms. It is possible that the extracts inhibited the TNF-α and IL-1β release by blocking a specific singling pathway. Macrophage stimulation causes the transactivation of numerous key transcription factors, including NF-κB, which regulate a variety of genes involved in inflammation. The NF-κB protein is found in the cytoplasm and binds to the protein inhibitor IkB in the absence of stimulation. Because IkB is degraded as a result of upstream inflammatory signals, NF-κB is able to translocate to the nucleus and control the downstream inflammatory response by binding to the kB site in the DNA structure. A canonical route is a well-known inflammatory signalling system. Inflammatory stimulation can activate MAPK pathways such as Extracellular Signal-Regulated Kinases (ERK), p38 MAPK, and JNK, in addition to NF-κB activation (cJun NH2-terminal kinases). Through the transactivation of AP-1 proteins, these three protein kinases also play a role in the regulation of genes linked to inflammation.

Overproduction of prostaglandin E2 (PGE2), nitric oxide (NO), and pro-inflammatory cytokines TNF-α and IL-1β characterise the inflammatory response. (Hotamisligil, 2006; Giriwono *et al*., 2019). Therefore, the ability of our *Cystoseira* extracts to block one or more certain elements in the singling pathways will lead to ant-cytokines release. This may happen via the decrease in the suppression of protein and mRNA expression of IL-1 and TNF-α and inhibition of MAPKs phosphorylation, especially the JNK pathway (Jayawardena *et al* 2019). In the previous study by Giriwono *et al*. (2019), ethanol extract from *sargassum patens* inhibited p65 of NF-κB translocation which has an important role in proinflammatory cytokines activation. It is also possible that the ability of extracts to reduce ROS formation may be involved in the inhibition of pro-inflammatory cytokines expression. This could explain the anti-inflammatory activity of extracts by suppressing NO production and iNOS expression, which may be associated with the attenuation of TNF-α formation in stimulated M1 macrophages-like cells. (Heo *et al*., 2010). The macrophages treated with the *Laminaria japonica* products inhibited the LPS-induced production of reactive oxygen species (ROS) and NF-κB (p65) phosphorylation, prostaglandin E2, and NO production in macrophages (Lin *et al*.,2016). In line with these studies, our results indicate that the anti-proinflammatory compounds in *Cystoseira* extracts tended to be responsible for the anti-TNF-α and IL-1β release activity through a certain signalling pathway. However, it has also been previously confirmed that algae products such as LPS-like compounds could prevent endotoxin shock and block sustained TLR4 stimulation required for cytokine expression (Macagno *et al.,* 2006). Our endotoxins assay (LAL) result showed that was not the case in our *Cystoseira* extracts as the extracts were not contaminated by endotoxins. Interestingly, the extracts showed to have anti-endotoxin activity as they blocked the LPS to approximately 50%. The mechanism of the extracts anti-endotoxin activity may be by LPS-neutralizing activity or LPS-binding, therefore, inhibiting the interaction of endotoxin with its receptors (De Tejada *et al*., 2015). Some bioactive compounds from brown seaweed, such as carrageenan, have the ability to change the macromolecular structure of LPS and prevent LPS from landing on the epithelial layer (Yermak *et al*., 2020). The result, therefore, suggests that the inhibition of TNF-α and IL-1β release from stimulated M1macrophage-like cells may be due to bioactive components in the extract blocking LPS from stimulating MI-like cells. However, endotoxin assay also showed that the extracts did not completely block the endotoxin LPS, which may suggest that the anti-proinflammatory effect happened via two mechanisms, preventing LPS from stimulating MI-like cells and blocking a specific singling pathway. Further supporting experiments were conducted, whereas the extracts and LPS were added together at the same time to the

M1macrophage-like cells did not show a notable difference in the inhibition of cytokines release from adding LPS in advance. This result means that the inhibitory effect is more likely due to the presence of active ingredients in *Cystoseira* extracts that have blocked a certain singling pathway of cytokine release.

The Gram-negative bacteria *Escherichia coli* is one of the most common causes of severe human illnesses that results from changing the conditions of systemic inflammation. Therefore, we were interested in studying whether our *Cystoseira* extracts have antibacterial activity against some pathogenic bacteria, including *E. coli.* Surprisingly, our *C. tamariscifolia*, *C. crinita* methanol extracts did not show any antibacterial activity against some pathogenic bacteria including *E. coli* compared to the standard antibiotic. Similar patrons of the result were reported by Maggio *et al*. (2020). Similarly, in a study by Lopes *et al*. (2012) *C. tamariscifolia*, the extract was less effective against both Gram-positive and Gram-negative bacteria despite presenting the highest polyphenol content among other *Cystoseira* species. The exact reason for this lack of antibacterial activity is unknown. We hypothesise that the choice of extraction solvent had a significant impact on the antibacterial characteristics of the extracts, implying that antimicrobial activity could be dependent on both algal species and extraction technique efficiency. It is also possible that bioactive compounds including polyphenols present in our *Cystoseira* extracts exhibit lower molecular weight or fewer hydroxyl groups free to react. *E. coli* bacteria produce multiple endotoxins that can be recognised by TLRs expressed on the surface of macrophages, and therefore, an initiated signalling pathway for releasing pro-inflammatory cytokines starts (Palmer *et al.,* 2011; Skjesol *et al.,* 2019). This shows that *E. coli* is a suitable model for stimulating macrophages for anti-proinflammatory studies. Therefore, we were interested in exploring the ability of anti-proinflammatory activity of the *Cystoseira* extracts on M1- like cells stimulated by heat-killed or Live *E. coli* strain K12 bacteria. However, before the anti-proinflammatory assessment, we examined the right concentration of the bacteria for stimulating M1 macrophage-like cells compared to the stimulation by LPS. Remarkably, when M1macrophage-like cells were treated with heat-killed or live *E. coli*, they demonstrated increased TNF-α and IL-1β release higher than when stimulated by LPS and LTA. This is in line with previous study where *E. coli* caused the release of TNF-α from murine macrophages (Paul-Clark *et al*., 2006). *E. coli* may activate TLR4 receptors via LPS which results in recruitment of MyD88 and MAL adapter proteins. TLR4 also recruits Toll/interleukin-1 receptor (TIR) domain-mediated cellular adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) adapter proteins. TNF-α is induced via the MyD88 pathway, whereas NOSII is induced via the MyD88-independent pathway via

TRIF/TRAM (Vogel *et al*., 2003). In general, live bacteria stimulated M1macrophage-like cells had higher levels of TNF-α and IL-1β secretion than heat-killed preparations, which came in line with previous findings achieved by Cross *et al.* (2004). This could be evidence for the existence of a variety of endotoxins and endotoxin-like compounds released from both *E. coli* that enhanced inflammatory activity. We also believe that the increase in the secretion of cytokines from the heat-killed or Live *E. coli* - stimulated macrophages did not result from the high concentration of LPS endotoxin alone in bacteria, as we have examined the equivalent bacteria concentration of 1 μg mL^{-1} of LPS and found it to be 10^9 mL^{-1} which we used for our experiments.

Next step, the heat-killed and live *E. coli* stimulated macrophage cells were exposed to *C. tamariscifolia* and *C. crinita* methanol extracts to examine their anti-TNF-α and IL1-β release activity.

The results were generally similar to the effect on both LPS and LTA stimulated M1 macrophage-like cells. The extracts significantly reduce TNF-α and IL-1β release from stimulated macrophage-like cells. Similar pattern of result was found by Eom *et al*., (2017) who studied the anti-inflammatory activity of bioactive compounds of brown seaweed *Eisenia bicyclis* on *Propionibacterium acnes*-induced human skin keratinocytes cells. The authors found that *P. acnes*–treated cells increased the expression of pro-inflammatory cytokines and chemokines and also found that of *E. bicyclis* bioactive compounds inhibited the expression or formation production of NO, and TNF-α. The authors revealed that the mechanism was via an inhibition in *P. acnes*-mediated phosphorylation of Akt and activation of NF-κB. In another study investigated the anti-inflammatory effect and the mechanism of seaweed polysaccharide on *E. coli*-stimulated porcine jejunal cell line IPEC-J2 model. The study showed that 4 h *E. coli* challenge, activated NF-κB signalling pathway and increased proinflammatory response, which indicates that the *E. coli* infection model was well-established. The study also demonstrated that the seaweeds products inhibited the activation of the NF-κB signalling pathway by 4 h *E. coli* challenge, decreased mRNA expression of TLR-4, MyD88, IκBα, p-65, as well as the reduced ratio of protein expression of p-p65/p65 which led to a decrease of the IL-6 and TNF-α release (Guo *et al*., 2021). However, in our study in the case of live *E. coli* stimulated M1macrophage-like cells, there was almost no inhibition of IL-1β release. The reason for this difference is unknown, and the collection of TLRs and other receptors involved in transducing the *Cystoseira* active compound toward the release of IL-1β may be less efficient. Signalling pathways can be regulated at various levels, from a receptor level to

transcription level via different mechanisms such as deubiquitination, proteolytic degradation and competition (Giambartolomei *et al*., 2004; Kanmani *et al*., 2019). *Cystoseira* active compounds may regulate the receptors pathway signalling by increasing their production, leading to the termination of the activation of receptors such as TLRs through negative feedback mechanisms.

In conclusion, we have demonstrated the anti-proinflammatory activity of the methanol extracts of *C. tamariscifolia* and *C. crinita* on the cytokines TNF-α and IL-1β release from stimulated M1macrophages-like cells. The outcome of the study reveals the extracts have potent anti-proinflammatory activity, and the extracts exhibited remarkable inhibition of TNF-α and IL-1β release from stimulated macrophages as well as found to have anti-endotoxin activity. These data suggest that *C. tamariscifolia* and *C. crinita* extracts may be therapeutic agents for the treatment of inflammatory diseases in the future. Purifying and identifying the specific component responsible for the anti-inflammation activity as well as understanding its biological properties and the mechanisms involved will be required for further pharmacological approaches. Taken together, it would be interesting to purify the component responsible for the anti-inflammation activity via the extract separation method using liquid mobile phases in High-Performance Liquid Chromatography. Besides, as a variety of natural products has been previously reported to have anti-proinflammatory activity, we think that it would be more interesting to identify the active component for future biological studies. Therefore, we next attempted to identify the compounds in the fraction that had anti-proinflammatory activity using the Mass Spectrum strategy (Chapter 5).

CHAPTER 5

ISOLATION AND IDENTIFICATION OF ANTI-INFLAMMATORY COMPOUNDS FROM *CYSTOSEIRA* SPP,

5.1 Introduction

Cystoseira genus is a relatively unexplored potential resource of new molecules for the pharmaceutical industry, in particular for the use as functional anti-inflammatory agents. Many bioactive molecules obtained from *Cystoseira,* such as polyphenols, polysaccharides, pigments and fatty acids, have demonstrated a wide variety of anti-inflammation activity (Olate-Gallegos *et al.,* 2019; Graiff *et al.,* 2016; Khan *et al.,* 2007). In the past, these bioactive compounds were analysed and measured by a colourimetric method which does not present a clear description of the quantity of the chemical composition in the algae extracts (Laurens *et al.,* 2012). Moreover, the vast diversity and complexity of the chemical composition present in the raw extracts make it necessary to process them for characterisation and isolation to obtain the active ingredients (Balboa *et al.,* 2013). Consequently, numerous advances in purification and analytical methods to classify such substances have been documented in recent years, providing valuable insights into the nature of the various biochemical structures of biologically active compounds (Demirel *et al.,* 2009; Kindleysides *et al.,* 2012; López *et al.,* 2011). A chromatographic technique in which high-performance liquid chromatography (HPLC) is involved has been the analytical procedure that has dominated the characterisation of bioactive compounds (Žuvela *et al.,* 2019). In this technique, the stationary phase is usually a column that contains silica or other polymers of tiny sizes, which leads to a large increase in the surface area and thus a significant improvement in the retention time. The mobile phase is usually one of the appropriate liquids, such as organic solvents (Liu *et al.,* 2002). Regardless of the difference between types of columns and mobile phases, C18 column and ultraviolet UV light detector have commonly been used on the chromatography separation to characterise, isolate and sometimes quantify bioactive substances from macroalgae. For instance, the total bioactive compounds yield is affected by the extraction solvent, polarity and molecular weight (Audibert *et al.,* 2010). Several techniques have been developed for identifying active compounds from natural sources using HPLC analysis (Khokhar & Magnusdottir, 2002; López *et al.,* 2011). For instance, phloroglucinol, gallic acid, catechin, rutin, gentisic acid, chlorogenic acid, caffeic acid, coumaric, ferulic, myricetin, quercetin, fucoxanthin and fatty acids were isolated from brown seaweeds crude extracts and identified using HPLC (Belda *et al* ., 2016: Liu *et al.,* 2012; Susanto *et al.,* 2016). However, owing to the high complexity and the unique structure of brown macroalgae bioactive compounds, A comparatively limited number of compounds have been effectively isolated or defined by HPLC alone. Therefore, a technique of advanced chromatography and mass spectrometry combined provide the option of further profound

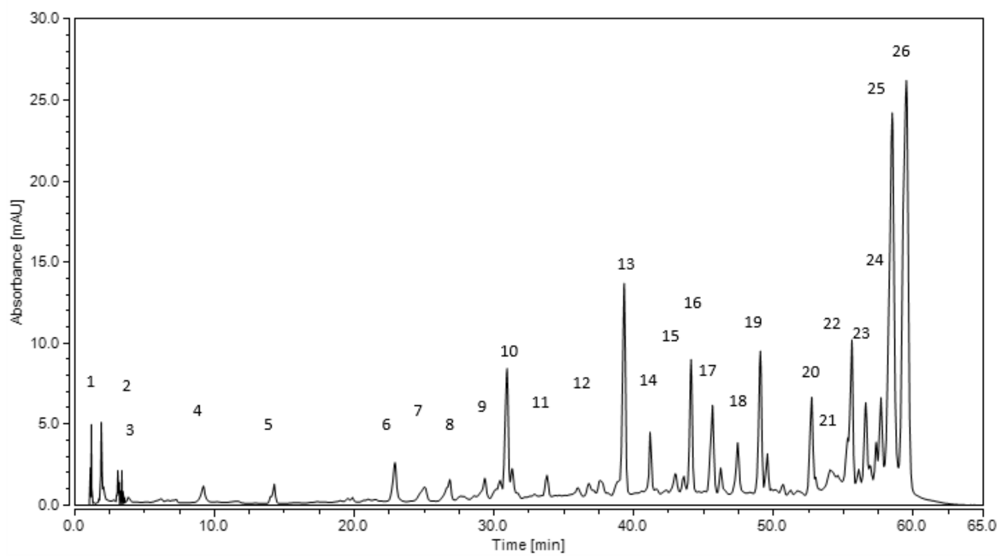
studies of bioactive compounds isomeric complexity. In particular, the use of HPLC for initial purification followed by Liquid chromatography-Mass Spectrometry (LC-MS) analysis is a beneficial method (Loos *et al.,* 2016). This analytical technique combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry helps to improve the resolution capacity of LC columns and the mass spectrometry scanning speed and sensitivity (Pitt, 2009). Several researchers have previously studied LC-MS analysis of bioactive compounds applications from various species of brown seaweeds. For instance, bioactive polyphenol profiles were characterised in extracts of three Chilean brown seaweeds, *Durvillaea antarctica*, *Lessonia spicata* and *Macrocystis integrifolia* and phlorotannins and flavonoid compounds identified in *D. antarctica* by LC-MS/MS (Olate-Gallegos *et al.,* 2019). LC-MS has also been used to identify fucoidan from *Fucus vesiculosus* (Rupérez *et al.,* 2002) and laminarin in *Laminaria digitata* (Graiff *et al.,* 2016). Three anti-inflammatory fatty acids, namely stearidonic acid, eicosapentaenoic acid and arachidonic acid, were isolated from *Undaria pinnatifida* and identified using MS analyses (Khan *et al.,* 2007). Fatty acids, including arachidonic acid, arachidic acid, palmitic acid, elaidic acid, linoleic acid and stearic acid, were isolated from *Sargassum thunbergii* and identified using chromatography-mass spectroscopy. They also significantly inhibited TNF-α release in stimulated human monocytic leukaemia (Kim *et al.,* 2010). Polyunsaturated fatty acids including eicosapentaenoic acid, eicosatrienoic acid, linolenic acid, and γ-linolenic acid were isolated from brown seaweeds *Ishige okamurae* and significantly reduced the release and the expression of interleukin IL-4 and IL-13 (Vo *et al.,* 2011). Taken together, this study demonstrates sophisticated chromatographic profiling and separation of *C. tamariscifolia* and *C. crinita* bioactive compounds, which showed potent anti-proinflammatory activity on stimulated M1macrophage-like cells. Methanolic extracts of *C. crinita* were profiled and then partitioned using HPLC grade water and methanol to obtain polaritic fractions. Fractions were tested for their anti-proinflammatory activity against the TNF-α release by LPS- activated M1macrophage-like cells. The result showed that one fraction from *C. crinita* had a significant anti-proinflammatory effect. LC-MS analysis of the fraction showed that the fraction contains fatty acids, namely, nonanedioic acid, undecanedioc acid, dihydroxy stearic acid, palmitic acid and oleic acid. The study suggests that *C. crinita* is a valuable source of anti-inflammatory agents, which may provide a source of the pharmacological industry.

5.2 Results

5.2.1 An initial HPLC chromatographic profiling of methanol extract of *Cystoseira* spp

As an initial step to purify the responsible substance for the anti- pro-inflammatory activity, a chromatographic analysis was performed using HPLC combined with a variable UV wavelength detector. The chromatographic elution of compounds with several compounds common among both samples is shown (Figure 5.1a and b). Overall, a total of 26 peaks were observed in *C. tamariscifolia* methanol extracts, all exhibiting a UV around 272 nm, where they were successfully separated. Most of the compounds present in *C. tamariscifolia* methanol extracts came at a lower polarity with the increase of the methanol mobile phase parentage and had lower molecular weights. *C. crinita* crude methanol extract displays a different profile to that observed for *C. tamariscifolia*. A total of 9 peaks were shown in *C. crinita* methanol extracts, all exhibiting a UV around 272 nm, where they were successfully separated. Similar to *C. tamariscifolia*, *C. crinita* crude extract shows that the majority of the compounds had broad molecular weights also the majority of the compounds present in *C. crinita* methanol extracts showed higher solubility in methanol.

(A)



(B)

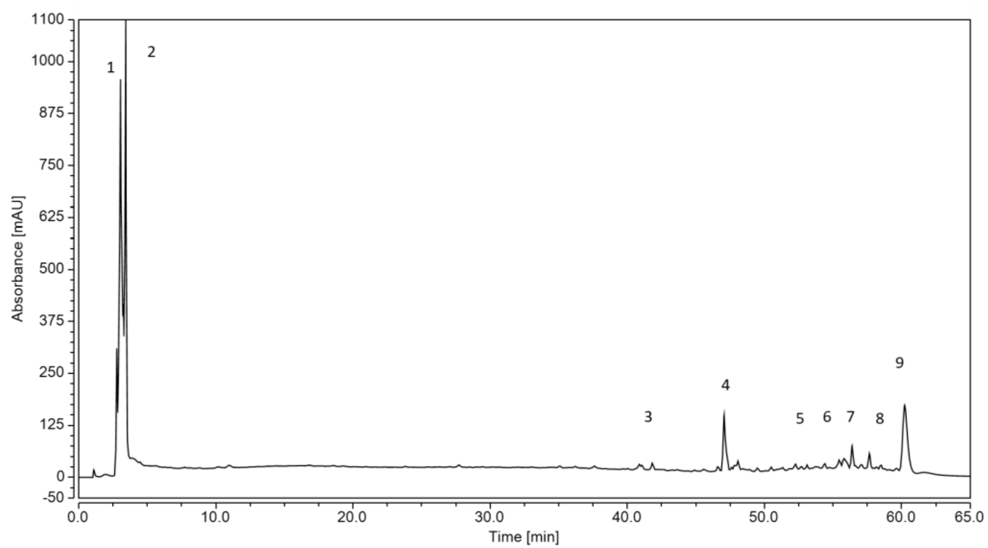


Figure 5.1 HPLC chromatogram of the methanol extracts of *C. tamariscifolia* (A) and *C. crinita* (B)

5.2.2 The effects of *Cystoseira* spp. methanol extract fractions on LPS-induced TNF-α release

To evaluate the anti-proinflammatory activity of the methanol extracts fractions of both *Cystoseira* species, fractions were added to media and LPS stimulated M1 macrophage-like cells at a final concentration of 250 µg mL^{-1} and incubated for 20 h. Supernatants were next collected, and ELISA was performed to evaluate the effect of fractions on the pro-inflammatory cytokines TNF-α release by stimulated M1 macrophage-like cells. ELISA results showed that the fractions T5 and T6 of *C. tamariscifolia* crude extracts have the most inhibitory effect on TNF-α release with approximately 50% to 40% reduction, respectively, as shown in figure (2). In contrast, fractions from T1 to T4 did not show an inhibitory effect on TNF-α release. Very different from *C. tamariscifolia,* fraction C1 from *C. crinita* methanol extract was significantly effective against TNF-α release from the stimulated macrophages with around 65% reduction. C3 fraction also showed an anti-proinflammatory effect with approximately a 40% reduction on TNF-α release, while other fractions did not show a notable effect on LPS-induced TNF-α release.

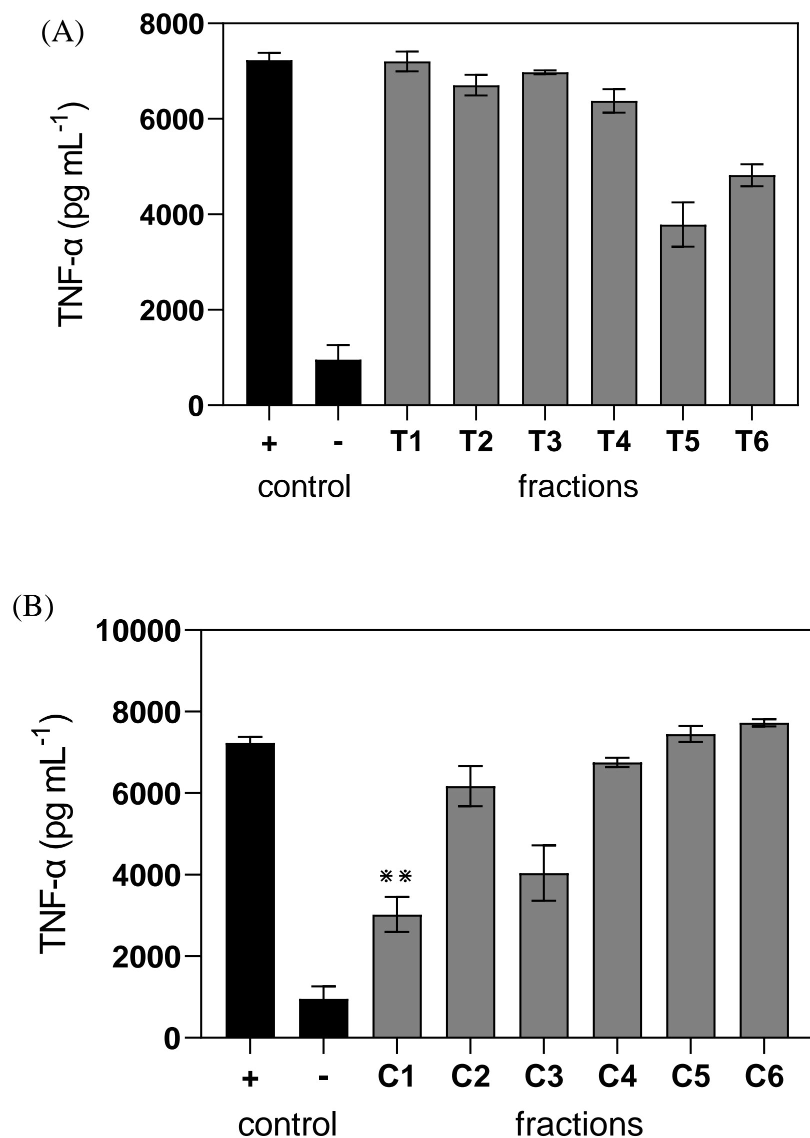


Figure 5.2 Effect of *C. tamariscifolia* (A) and *C. crinita* (B) methanol extract fractions on TNF-α in LPS-stimulated M1 macrophage-like cells. TNF-α released into the culture medium was assayed by the ELISA method. The data represent the mean ± SD of triplicate experiments. * P<0.05 vs LPS alone.

5.2.3 The effect of sup-C1 fractions of *Cystoseira crinita* on LPS-induced TNF-α in M1macrophage-like cells

For further purification of the anti-proinflammatory active compounds in the C1 fraction from *C. crinita,* which had the highest inhibitory effect on the TNF-α release, further separation of the C1 fraction was performed under similar chromatographic conditions that in HPLC extracts initial fractionation. As shown in figure (5.3), the C1 fraction was separated into new three fractions C1a, C1b and C1c, by collecting eluting from the column every 3.3 minutes. In order to obtain a high concentration of the active component, which allows conducting replicated biological experiments, the C1 fraction separating process was repeated 20 times, and the eluting fluid was concentrated.

The resulted fractions C1a, C1b and C1c, were added to media and cells to evaluate the inhibitory effect of fractions on LPS-induced TNF-α in M1macrophage-like cells using ELISA. The result showed that the fraction C1c is the fraction that contains the most active components, which significantly reduced the TNF-α release compared with the control by approximately 80%, as shown in figure (5.3). Fraction C1a and C1b showed lower anti-TNF-α release with around 50% and 40% reduction. It is worth mentioning that the solvent effect was examined in all anti-proinflammatory experiments and did not show any effect on cytokines release. The M1 macrophages-like cells were exposed to fractions and incubated for 20 hours at 37 °C in a humidified atmosphere containing 5% CO_2. As illustrated in figure (5.4), the MTT data indicate that the fractions did not show a cytotoxic effect on M1 macrophage-like cells as well as did not show a cytotoxic effect (figure 5.5).

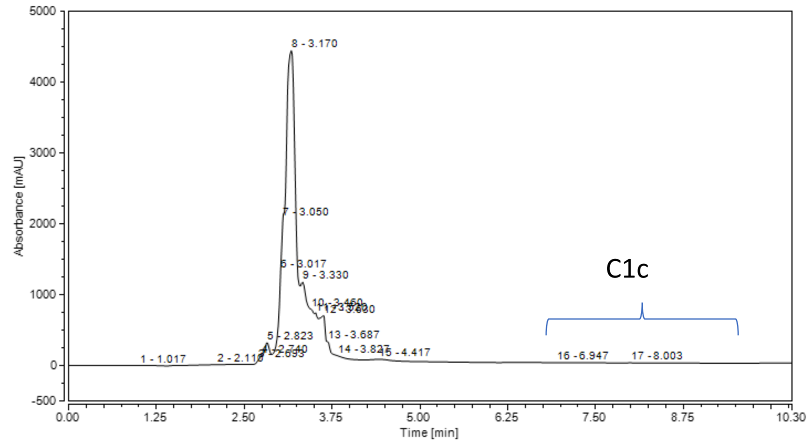


Figure 5.3 HPLC profile of the active anti-proinflammatory fraction C1 from *C. crinita* extract

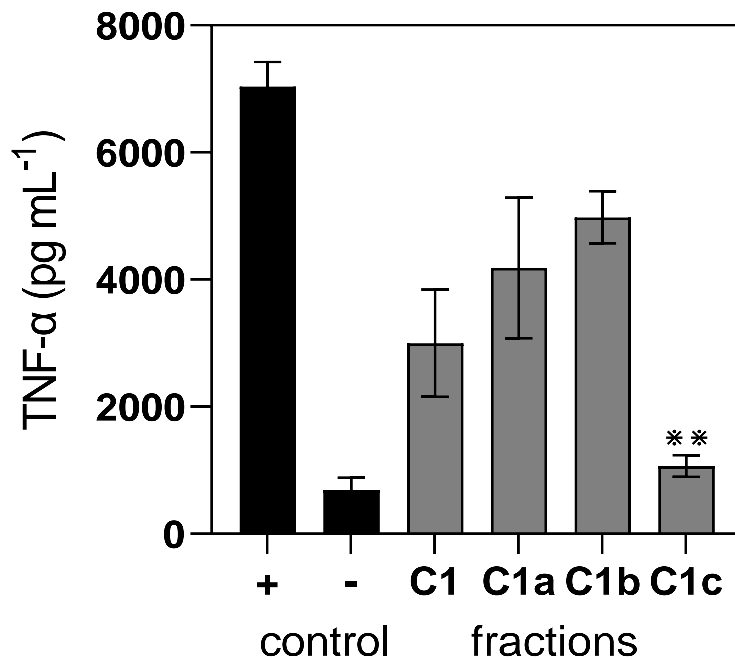


Figure 5.4 Effect of *C. crinita* extracts fractions C1, C1a, C1b and C1c on TNF-α in LPS-stimulated M1 macrophage-like cells. TNF-α released into the culture medium was assayed by the ELISA method. The data represent the mean ± SD of triplicate experiments. * $P<0.05$ vs LPS alone.

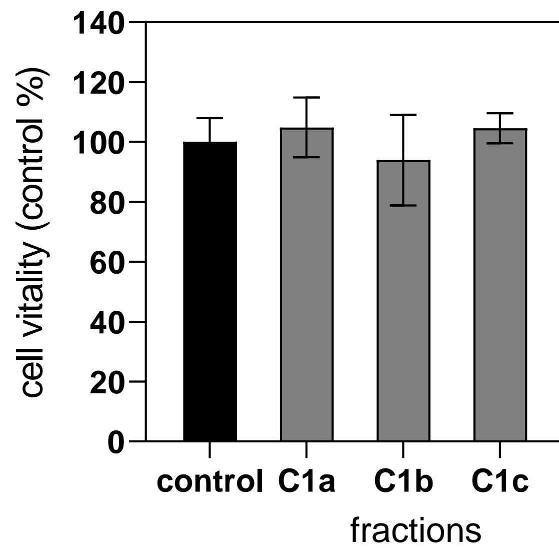


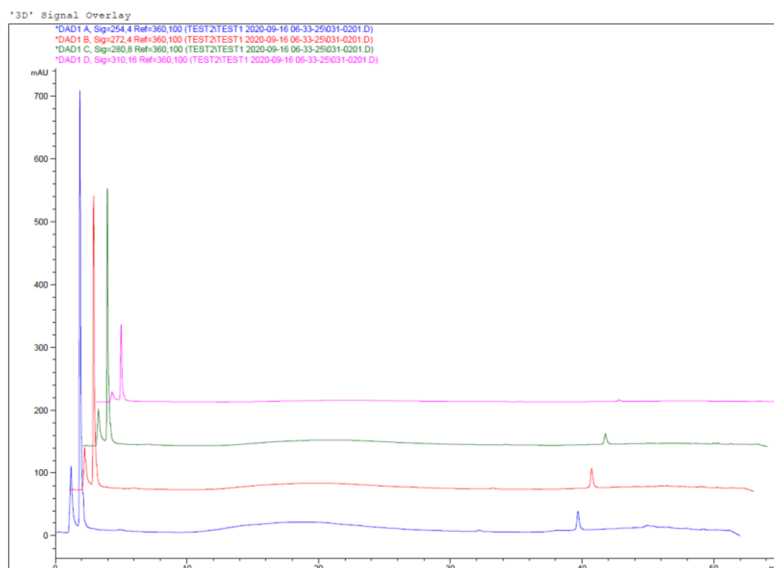
Figure 5.5 Percentage of cell vitality in comparison to control (methanol) (mean ± SD; n=3). Macrophage-like cells were exposed to C1 fractions of *C. crinita* for 20 h.

5.2.4 The identification of anti-proinflammatory compounds in fraction C1c from *Cystoseira crinita* among the HPLC–DAD–ESI/MS (positive and negative ion mode)

HPLC with UV-Vis diode array detection DAD was used for chromatographic analysis of the active anti-proinflammatory fraction C1c. 50 µl of C1c fraction, which was collected repeatedly between minutes 7 – 10 in the initial HPLC analysis, was analysed directly by LC/UV. As observed in the figure (5.6. A, B), C1c homogenates only contained three peaks in the 2 - 39 minute stage. Sharp peaks of interest were observed between 2 and 7 mins and a small peak at about 39mins.

LC/ESI–MS was applied for the analysis of the anti-proinflammatory active fraction C1c from *C. crinita* by the determination of the molecular mass of each peak. As the chemical properties of the putative components of interest in the sample are unknown, an analysis was carried out under similar optimised conditions in positive and negative ion mode. In the positive ion analysis and before background subtraction, the LC/ESI-MS chromatograms for positive ion mode LC/ESI-MS analyses were similar to the LC/UV data, with evidence of components of interest beyond a number of peaks around 2-2.5 mins and later eluting peaks at a high percentage of MeOH. After background subtraction by the blank MeOH, low-intensity peaks were noted throughout the chromatogram (figure 5.7. A, B). The accurate m/z values of the ions of interest in these peaks were used to generate lists of potentially matching elemental formulae, with C, H, N, O, Na and occasionally S used as the incorporated elements only. The main peak at around 2-2.5 mins is consistent with the presence of Mannitol, and most peaks could not be assigned without further evidence. Therefore, a negative ion analysis was conducted, which showed fewer obvious peaks generated by ions of lower intensity even after background subtraction. The accurate m/z values of the ions of interest in the peaks observed were used to generate lists of potentially matching elemental formulae, with C, H, N, O, Na and Cl (when relevant isotopes observed) used as the incorporated elements only and are useful as a starting point to search PubChem and other databanks for potential matches (Table 5.1). The main peak at around 2-2.5 mins is again consistent with the presence of mannitol, and later eluting peaks correlate with fatty acids, namely, nonanedioic acid, undecanedioc acid, dihydroxy stearic acid, palmitic acid and oleic acid (figures 5.8 a, b, c, d and E)

(A)



(B)

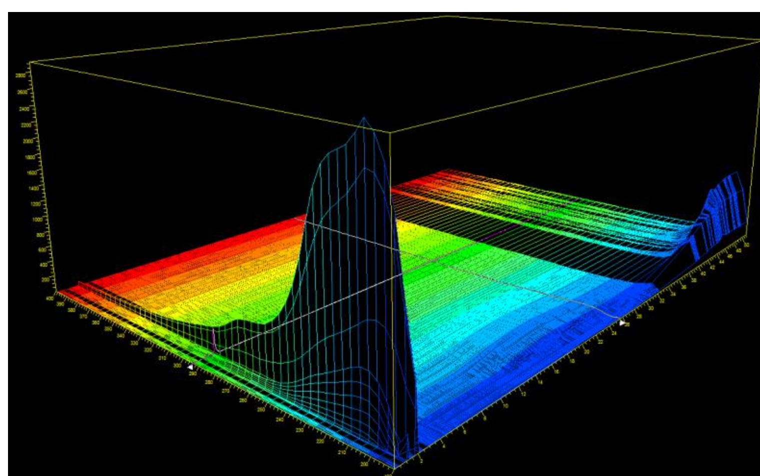
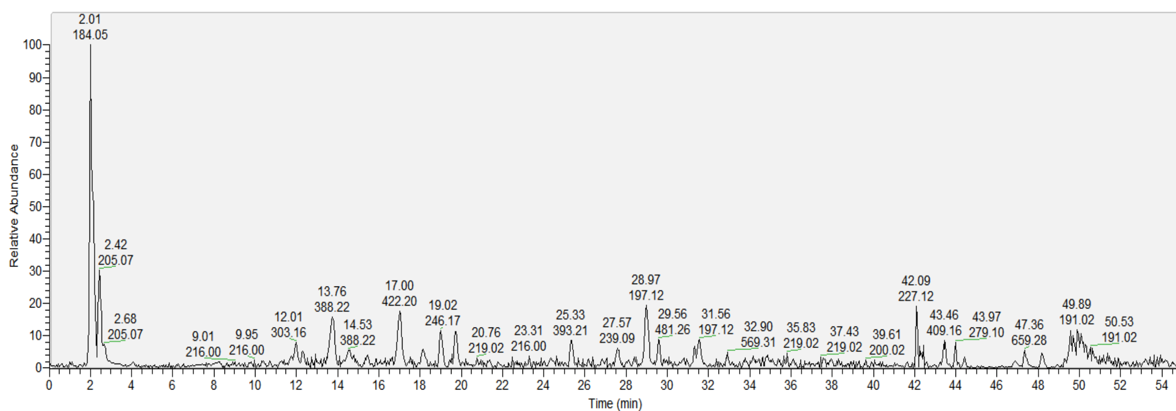


Figure 5.6 L.C./UV-Vis chromatogram (A) and 3-D DAD spectrum (B) of C1c fraction from *C. crinita*

(A)



(B)

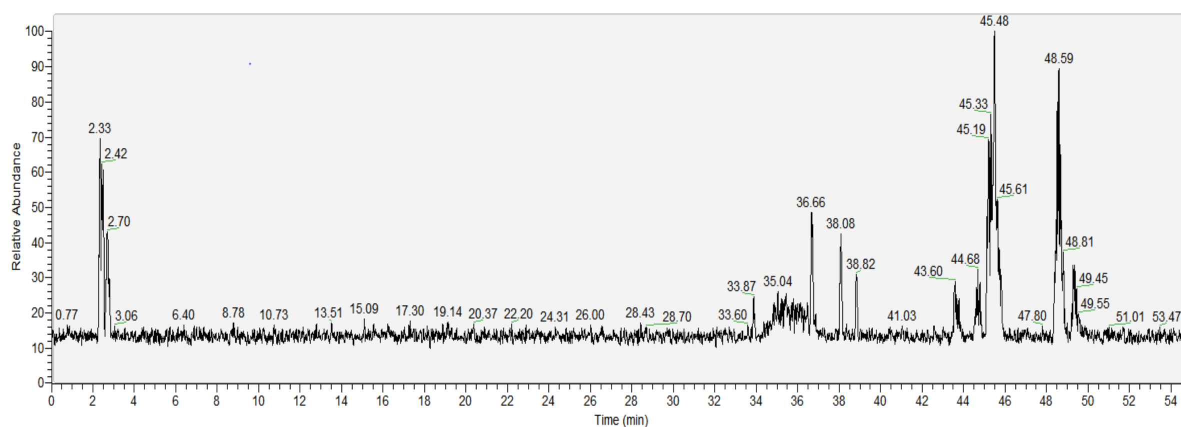


Figure 5.7 LC/ESI–MS background subtracted base peak chromatogram of C1c fraction in both Positive Ion LC/ESI-MS Analysis (A) and Negative Ion LC/ESI-MS Analysis (B).

Table 5.1 Identification of the active anti proinflammatory compounds Mannitol and fatty acids in C1c fraction of *C. crinita* observed by HILIC–ESI–MS, as positive ions mode and negative ion mode showing their molecular mass, retention time and molecular formula.

			Predicted Ions (m/z)		Observed Ions (m/z)	
	Potential Molecules	Formula	[M+H]+	[M+Na]+	Rt (mins)	C1c
C1c LC-MS positive ion analysis	Mannitol	$C_6H_{14}O_6$	205.0683		2.4	205.0683
C1c LC-MS negative ion analysis	Mannitol	$C_6H_{14}O_6$	217.0484		2.3	217.0486
	Nonanedioic acid	$C_9H_{16}O_4$	187.0976		26.5	187.0981
	Undecanedioc acid	$C_{11}H_{20}O_4$	215.1289		33.0	215.1291
	Dihydroxy stearic acid	$C_{18}H_{36}O_4$	315.2541		41.4	315.2541
	Palmitic Acid	$C_{16}H_{32}O_2$	255.2330		43.8	255.2332
	Oleic Acid	$C_{18}H_{34}O_2$	281.2486		44.8	281.2487

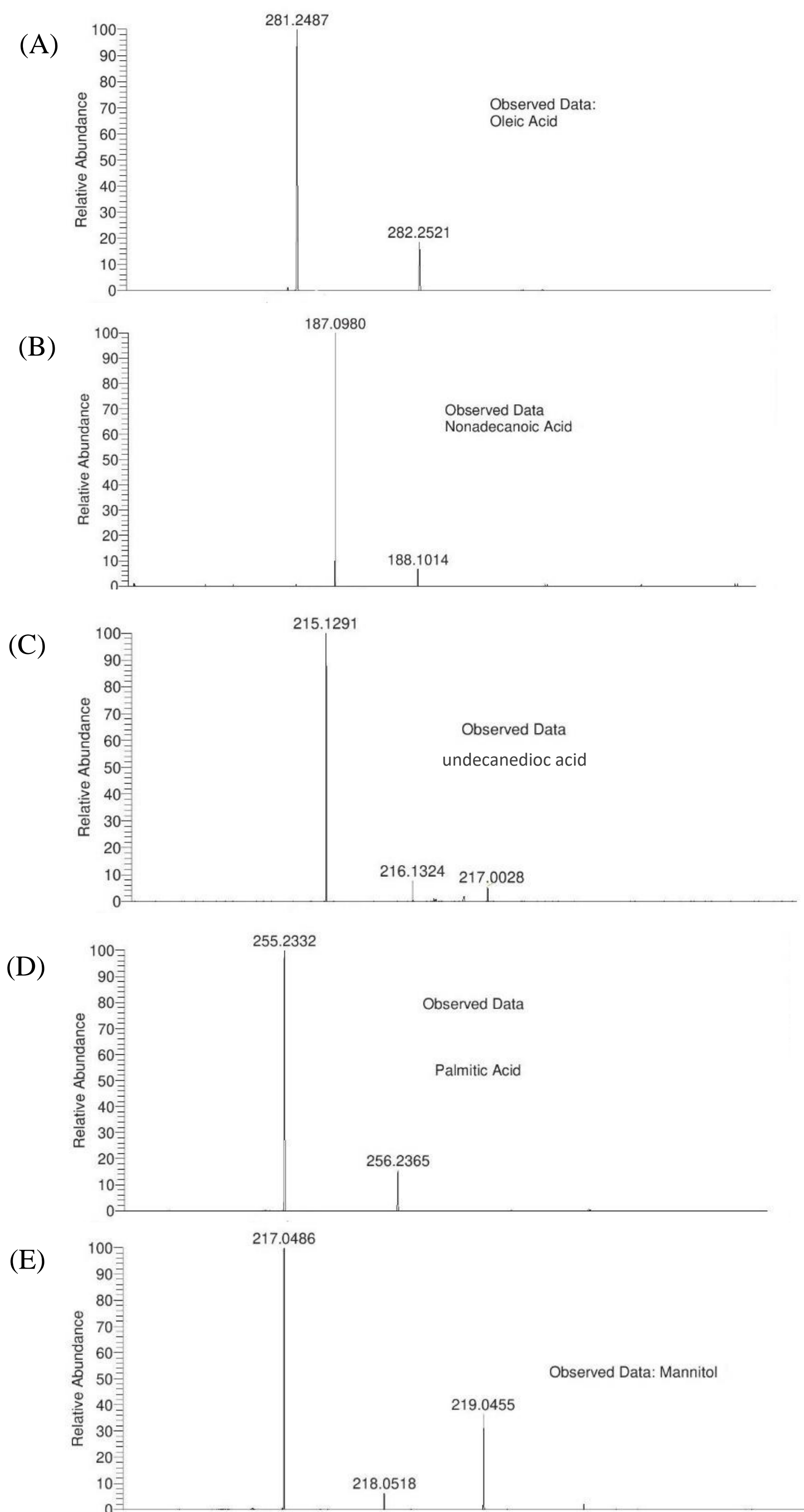


Figure 5.8 Mass spectrum of fatty acids and mannitol appeared in the active proinflammatory fraction C1c from *C. crinita*. (A) oleic acid, (B) nonanedioic acid, (C) undecanedioc acid, (D) palmitic acid and (E) mannitol.

5.3 Discussion

Numerous structurally unique and biologically active substances of marine organisms are identified and are under review or are being produced as potential pharmaceuticals. Among marine organisms, brown macroalgae are well known as a rich source of natural bioactive molecules, some of which have been proven to display anti-inflammatory activity, including polysaccharides, polyphenols, steroids and carotenoids (Abad *et al.,* 2008). As demonstrated in the previous chapter, the crude methanol extracts of the brown seaweeds *C. tamariscifolia* and *C. crinita* methanol were found to have a potent anti- pro-inflammatory activity. However, in the case of drug discovery efforts, purifying and identifying important pharmacological ingredients as a drug in the laboratory is usually the ultimate goal. Crude drugs from the natural provider are facing challenges such as cost, time, organisational, and even scientific views on studying the experimental molecules of raw drugs in humans (Lahlou, 2007). Advanced isolation tools and *in vitro* tests are therefore used to identify the bioactive chemical components of the raw drug that is expected to have a specific therapeutic effect in humans. This provides a rational basis for standardising crude drug formulas and isolated compounds for screening to humans.

The HPLC chromatographic profiling has been shown to be an important method for substitution in crude extracts of medical marine organisms (Wolfender *et al.,* 2010). This analytical method can produce the fingerprint of crude extract should be helpful in detecting bioactive compounds in a vast number of compounds. In this study, we attempted to compare the HPLC fingerprints of methanolic extracts of brown seaweeds *C. tamariscifolia* and *C. crinita* as well as we searched for effective anti-inflammatory compounds in the crude methanol extracts. A normal phase Silica-A column with a solution of water and methanol combined with ultraviolet detection at 270-280 nm provided good separation. Through gradient mode, the volume of one of the solvents decreases over time, making it a better option for the study of complicated unknown mixed samples (Ahuja & Dong, 2005). Due to the sharpness of the peaks and proper baseline, HPLC analysis of *C. tamariscifolia* and *C. crinita* extract showed the presence of various constituents as evidenced by the chromatogram at different retention times. HPLC profile shows the elution pattern of compounds, and also a good separation of peaks was achieved. Our results showed that all peaks were sharp and baseline resolved. This means the conspicuous initial mobile phase concentration peak and a shallow gradient were able to provide good quality separation of the reagents and extracts. A similar result of HPLC extracts profiling was previously reported in several studies in a number of

brown macroalgae species (Jégou *et al.,* 2010; Andrade *et al.,* 2013). In the case of *C. tamariscifolia,* crude extract peaks appeared along with the chromatogram; however, more peaks appeared with the increase of the methanol mobile phase percentage. This means the majority of extract compounds are less polarity and mostly water-insoluble than those that appeared at the beginning of the chromatograph when high parentage of water. In *C. crinita* similar profile was observed, except a huge peak appeared at the high percentage of water mobile phase. This difference between the chromatographic profile of *Cystoseira* species could be explained by the big difference in the environmental conditions between the two species locations, which may affect the metabolic mechanism that resulted in big differences in compounds concentrations. For instance, *C. tamariscifolia* tends to minimise damage from environmental conditions by a combination of thallus thickness and increase of secondary metabolites production as a response to temperature increase and high light conditions, which might give an advantage over other species (Heavisides *et al.,* 2018; Mannino & Micheli, 2020).

HPLC profile was next developed to be validated for the fractionation of extracts and isolating the active anti-proinflammatory compounds. Higher concentrations of extracts and a bigger injection volume were used to obtain a high concentration of the bioactive compounds in the fractions. Six fractions resulted from each species sample and then were concentrated. Our result showed that purified methanol extract fractions T5, T6 of *C. tamariscifolia* and C1 and C3 from *C. crinita* exhibited anti-proinflammatory activity in LPS-stimulated M1 Macrophage-like cells. A similar result was reported by Mhadhebi *et al.* (2011) and Mhadhebi *et al.* (2012), who investigated the anti-inflammatory activity of methanol fractions from Mediterranean brown seaweed *C. sedoides* and *C. compressa*. As the C1 fraction from *C. crinita* showed the highest inhibition effect of TNF-α release as well as found to contain one peak, it was chosen for further purification and next identification. The fraction was separated into three fractions C1a, C1b and C1c, by collecting column eluent every 3.3 minutes. We next tested the potential anti-inflammatory activity of a purified fraction of *C. crinita* (C1a, C1b and C1c) on stimulated M1 macrophage-like cells. The ELISA result showed that C1c purified fraction significantly inhibited the expression of pro-inflammatory cytokines TNF-α from stimulated M1 macrophage without showing cytotoxic activity.

LC-MS is a powerful and useful technique used to identify functional groups, and further studies through this path could help in the structure elucidation of certain pure compounds. LC-MS is easy to use and is applicable for a substantially larger number of relevant analytes. With

the development of LC-MS, the widespread application of the proven principle of isotope dilution mass spectrometry is now possible not only in research but also for routine applications (Vogeser & Parhofer, 2007). This research used an LC/ESI–MS analysis flowing NHMR method to characterise and identify the bioactive compounds in the fraction C1c of *C. crinita* that responsible for the reduction of the TNF-α release in the stimulated macrophage. The result revealed the presence of fatty acids, including nonanedioic acid, undecanedioc acid, dihydroxy stearic acid, palmitic acid and oleic acid as well as mannitol. A similar group of fatty acids were reported in *C. crinita* from the black sea using the GC-MS technique (Ivanova *et al.,* 2013). The presence of fatty acids in the *C. crinita* extract also corroborated well with earlier reports in *Cystoseira barbata*, *C. nodicaulis*, *C. tamariscifolia*, C. usneoides, *C. abies-marina*, *C. crinita*, *C. humilis* and *C. osmundacea* (Duarte, 2016). Despite that macroalgae have low lipid content, among them, *Cystoseira* has the higher lipid contents, particularly saturated fatty acids, which represent almost half of this lipid content with a significant amount (MacArtain *et al*., 2007). For instance, palmitic acid was previously reported to be found in *C. compressa*, *C. barbata* and *C. nodicaulis* in *C. barbata*, *C. tamariscifolia*, *C. usneoides*, *C. abies-marina*, *C. crinita* and *C. osmundacea* (Khotimchenko *et al*., 2002; Prikha *et al*., 2011; Ivanova *et al*., 2013; Patarra *et al*., 2013; Silva *et al*., 2013). Similarly, monounsaturated fatty acids such as palmitoleic acid and oleic acid were also detected in *C. humilis, C. baccata*, *C. compressa*, *C. barbata*, *C. abies-marina*, *C. nodicaulis*, *C. tamariscifolia*, *C. usneoides C. crinita* and *C. osmundacea* (Patarra *et al*. 2013; Ivanova *et al*. 2013; Khotimchenko *et al*. 2002).

In our study, the fatty acids that have been found in the *C. crinita* anti-proinflammatory active fraction and then identified have been already recognised to have wide health benefits, and some of them are already prescribed as a drug including the anti-inflammatory effect. Nonanedioic acid, also known as azelaic acid, is a medium-chain fatty acid found in some grains and animal products. It is a fatty acid with an aliphatic tail that has between 4 and 12 carbon atoms(National Centre for Biotechnology Information, 2020). Nonanedioic acid possesses several bioactivities, including a direct anti-inflammatory effect due to its scavenger activity of free oxygen radicals and is used for the topical treatment of mild-to-moderate inflammatory acne vulgaris (Jones, 2009). Undecanedioic acid, also known as 1, 11-undecanedioate, belongs to the class of organic compounds known as medium-chain fatty acids with two carboxylic acid groups at positions C-1and C-9. It has a role as a metabolite and has been detected in multiple biofluids, such as faeces, saliva, and urine (National Centre for Biotechnology Information, 2020). Palmitic acid or hexadecanoic acid is the most common saturated fatty acid found in animals, microorganisms and plants, especially palms, as it is the

main element in palm oil (Carta *et al.,* 2017). Palmitic acid has an important effect on stimulating innate immune cells to regulate their inflammatory responses. In fact, palmitic acid upregulates interleukins IL-6, IL-8, TLR2. It also induces M1 macrophage polarisation through reducing chemokine and pro-inflammatory cytokine expressions, such as IL-6 and TNF-α. Moreover, palmitic acid also has an impact on the production of TLR4 and NF-κB-mediated pro-inflammatory regulators through the receptor-interacting protein 3 (RIP3) inflammasome activation, leading to macrophage-associated inflammation (Tzeng *et al.,* 2019; Kawa *et al.,* 2019).

Oleic acid or cis-9-octadecenoic acid is an omega-9 fatty acid, and it is the main fatty acid in olive oil and can be found in other vegetables and used as a source of fat in the diet and as a replacement for animal fat (Choi *et al.,* 2010). Oleic acids, usually known as neutral or pro-inflammatory fatty acid, and it has been reported to inhibit LPS-induced TNF-α secretion in mouse macrophage by inducing TNF-α gene expression under basal and inflammatory conditions (de Lima-Salgado *et al.,* 2011). However, conversely, the oleic acid increased stimulated the production of TNF-α and IL-8 levels as well as IL-6, IL-1β, and the chemokine neutrophils. Hence, oleic acid induces the synthesis of the main inflammatory mediators involved in clinical trials (Ballard-Croft *et al.,* 2012; Gonçalves-de-Albuquerque *et al.,* 2012). In fact, many previous studies have shown that fatty acids can decrease TNF-α production both *in vitro* and *in vivo* (Wang & Huang, 2015; Li *et al.,* 2019; Calder, 2006; Fontaine-Bisson *et al.,* 2007). Consistent with these observations, our research using the LPS-stimulated M1 macrophage-like cells showed that the release of TNF-α was significantly reduced. Most likely that fatty acids had the ability to reduce the release of TNF-α in LPS stimulated immune cells by preventing activation of NF-κB. For instance, TNF-α activates NFkB, which consequently induces TNF-α, driving to the increase of PGE2 release, and so more stimulation of NFkB by PGE2 (Calder, 2006). Because NF-κB appears to be a key factor in mediating most inflammation mechanisms, the inhibition of NF-κB may be the crucial point for the effects of the fatty acids on cytokine release and inflammation (Weldon *et al.,* 2007). The inhibitory effect on cytokine release might also be as a result of the changes in the formation of the lipid mediators, or the effect of the fatty acids on inflammatory genes expression as well as increasing anti-inflammatory cytokines such as IL-10 and IL-4 (Calder, 2006; Pereira *et al*. 2012).

Our LC/ESI–MS analysis data also showed that the C1c fraction from *C. crinita* contains the mannitol in both positive and negative MS mode. Mannitol is a common sugar in brown seaweeds and has also been detected in *C. crinita* for decades ago in many studies (Amico *et*

al., 1976; Usov & Chizhov, 1993; Chizhov *et al.,* 1998). However, in relatively recent time, Yalçın *et al.* (2002) did not find any free monosaccharides and mannitol in the Mediterranean Sea *C. crinita*. Mannitol is a major soluble storage carbohydrate in the Phaeophyta virtually ubiquitous with concentrations of approximately 5-25% of dry weight (Drew, 1969). It belongs to the class of organic compounds known as sugar alcohols which are hydrogenated forms of carbohydrate. Mannitol is used widely as a drug for a number of diseases such as renal failure and cerebral oedema (National Center for Biotechnology Information, 2020). However, very few studies have been conducted on mannitol isolated from the brown seaweed as a possible anti-proinflammatory agent taking into consideration the unique structure of brown seaweed metabolites. Some studies have been conducted on mannitol from other resources have reported that mannitol has a possible anti-NTF-α release effect. For instance, in a study conducted by Schreibman *et al.* (2018), Mannitol showed to reduce neuroinflammation in ischemic and traumatic brain injury in rat models. It is believed that mannitol reduced the markers of the macrophage M1macrophage-like cells phenotype (nuclear p65, TNF, and NOS2) and reduced the infiltration of CD45 + cells. Similarly, mannitol also significantly reduced the effects of TNF-α against hearing cells by inhibiting c-Jun N terminal kinase (JNK) activation pathway (Infante *et al.,* 2012). On the contrary, mannitol also seems to have a tremendous pro-inflammatory effect in some cases as it has shown to increase in TNF-α Release From LPS-Stimulated Feline PBMC (Morohoshi *et al.,* 1996; Haak & DeClue, 2008).

Taking together, as the previous studies suggested that both fatty acids and mannitol have the ability to inhibit the release of TNF-α from immune cells, including macrophages, we suggest that the effect of inhibition of the TNF-α release may be due to the action of all the compounds together. Since TNF-α is an important factor in many inflammatory extreme reactions that result in many immune diseases, the abundant availability of *C. crinita* along the Libyan coastline opens up a new avenue for the entry of pharmaceutical industries in developing anti-proinflammatory drugs. Future work includes more advanced separation methods as well as investigating the mechanism of the active anti-proinflammatory compounds that may help to reach A certain compound that has the most effective anti-TNF-α release.

CHAPTER 6

GENERAL DISCUSSION

6.1 **Discussion**

This study is a reported investigation into the anticancer and anti-inflammatory activity of extracts and compounds sourced from the brown macroalga *Cystoseira tamariscifolia, C. crinita* and *C. compressa* collected from The U.K. and Libya coasts. As reviewed in Chapter 1, several previous studies have demonstrated that brown seaweeds, in particular, the *Cystoseira* genus might be promising sources of anticancer and anti-inflammatory compounds (Mhadhebi *et al.,* 2011; Vizetto-Duarte *et al.,* 2016; Zbakh *et al.,* 2020). However, the researchers are very scattered and limited in scope and depth, especially targeting multiple extraction methods as well as the seasonality effect on the chemical composition and anticancer activity. In fact, this study for the first time explores and compares the potential cytotoxic activity of *Cystoseira* species collected from the U.K. and Libya in leukaemia, prostate and lymphoma human cancer *in vitro*, as well as the comparison of the effect of extraction method and seasonality on the cytotoxic activity of *Cystoseira*-derived bioactive substances. *Cystoseira* extracts were prepared from freshly harvested samples and subjected to coulometric measurements and next to *in vitro* cytotoxic assays as described in Chapter 3. A comparison of the content of the bioactive compound between the three *Cystoseira* species revealed the highest content of total polyphenol, flavonoids, polysaccharides and protein in *C. tamariscifolia*. Similar observations have been reported previously in Portuguese *C. tamariscifolia* (Nwosu, 2011). This may imply a type-specific sensitivity to environmental factors. It is known that active biological compounds often act as chemical protection compounds against extreme environmental conditions (Michel & Macfarlane, 1996). For instance, the great difference between the hours of day and night in the English Channel region, where *C. tamariscifolia* were collected compared to the Mediterranean region, where both *C. crinita* and *C. compressa* were collected, may be an important factor in the presence of a greater amount of bioactive compounds in *C. tamariscifolia* extracts. Our study also shows that methanol and chloroform extracts of the three *Cystoseira* species have the highest concentration of the total bioactive composition. Duarte (2016) previously reported a similar observation. This indicates the variety in the polarity of the compounds, which show *Cystoseira* value as a source of a wide range of bioactive compounds. In the case of *C. tamariscifolia*, seasonality has also shown an effect on the total bioactive compounds yield. In general, the summer and spring seasons had a higher impact of encouraging *Cystoseira* to produce a higher concentration of bioactive compounds. These results are in agreement with (Celis-Plá *et al.,* 2016) findings. However, in a study conducted by Duarte (2016) in Spain, *C. tamariscifolia*

extracts were found to have the highest concentration of biochemical composition in winter. This suggests that locations and environmental conditions are crucial factors for collecting samples to achieve the highest possible level of bioactive compounds from *Cystoseira.*

As *Cystoseira* crude extracts showed to have a considerable level of bioactive compounds, we aimed to evaluate the cytotoxic effect of the crude extracts of *C. tamariscifolia, C. crinita* and *C. compressa* on HL60, THP-1, PC3 and REC-1 cell lines which represent some of the most common cancer diseases that cause millions of lives lost every year worldwide (Fitzmaurice *et al.,* 2017). Here we show that within the three *Cystoseira* species tested in the current study (Chapter 3), methanol and chloroform extracts had the highest cytotoxic activity IC_{50} ranged between 2.32 ± 0.21 and 6.04 ± 0.9 µg mL^{-1}. Similar findings have been reported by (Abourriche *et al.,* 1999; Ahmed *et al.,* 2020). However, water extracts showed moderate cytotoxic activity against PC3, which indicates that the wide variety of cytotoxic compounds in the crude extracts ranged from non-polar to high polar extracts. This also shows that the cytotoxicity effect of the extracts may differ based on the cancer cell type as suspensions cells were more acceptable for the methanol and chloroform crude extracts. In contrast, the adhesive PC3 cells were more acceptable to water extracts and more resistant to methanol and chloroform extracts in most cases. Since there was a seasonality effect on the chemical composition of *C. tamariscifolia* reported earlier in this study, we suggested that seasonality may influence the cytotoxic effect as a result of the seasonal variation of the biochemicals. Here, for the first time, we demonstrated that the seasonality might also affect the cytotoxicity of the crude extracts of *C. tamariscifolia* against HL60, THP-1, PC3 and REC-1. In general, this study shows that summer and spring extracts have the highest inhibition in cell vitality of all examined cancer cells. There were, however, some exceptions based on the extraction method and the type of cancer cell lines; for example, winter extracts had a high cytotoxic effect against the PC3 cell line, unlike other cells. Considering this and the heterogeneity of cancer cells and the consequent marginal response to the natural anticancer products (Min *et al.,* 2019), the cytotoxic effect of each extract should be evaluated on a specific human cancer cell line separately and should not be generalised.

As discussed in the introduction chapter, it is known that different classes of biochemical compounds extracted from brown seaweeds, in particular, *Cystoseira,* have cytotoxic activity. Here we tried to figure out which class of bioactive compounds was responsible for the cytotoxicity activity. Interestingly, a positive correlation was found between the concentration of total content polyphenols of the *Cystoseira* extracts and the anticancer effect. The high levels of TPC led to low values of IC_{50} which means high cytotoxicity activity against HL60, THP-1

and PC3 cancer cell lines . This could explain the cytotoxicity activity of the extracts where the high concentration of phenolic content in the extracts were able of acting as cytotoxic agents and might be supported by the best possible conditions offered throughout the extraction procedure, which is beneficial for the extract's anticancer activity. This correlation was also observed also between the cytotoxic effects of *C. tamariscifolia* against human neuroblastoma in a study by Moussa *et al.* (2020). However, in our study, the positive correlation was not highly strong, which might also suggest that various bioactive compounds may be present in the extracts and that their relative amounts may vary with both season and extraction methods. In the case of phenolic compounds, it is also necessary to consider the collecting duration in addition to the extraction conditions. As is well known, phenolic compound synthesis is mostly influenced by environmental factors such as the presence of grazers and direct sunlight exposure. As a result, the content may fluctuate according to the year seasons (Mhadhebi *et al*., 2014: Bravo, 1998). At this point, however, it was not clear how *Cystoseira* extracts exhibited the anticancer effect on the cancer cell lines. It has been reported that bioactive compounds derived from brown seaweeds have shown deferent mechanisms of action in inducing cytotoxicity in cancer cell lines, including binding to various cell sites, suppressing cell divisions, or inducing apoptosis, topoisomerase inhibition and cell-cycle arrest (Lichota & Gwozdzinski, 2018; Saadaoui *et al.,* 2020). Therefore, for further investigation of the anticancer activity of the *Cystoseira* spp, we conducted fluorescent staining as an attempt to understand the cytotoxic mechanism of methanol extract of *C. tamariscifolia* on both HL60 and THP-1 cancer cell lines. Our result showed that the extracts exhibited nuclei fragmentation and induced apoptosis compared with control which also supported the observations of the cytotoxic activity of the crude extracts (chapter 3). However, in general, this study of the cytotoxic activity of the *Cystoseira* spp extracts suggests that extracts may provide a source of natural anticancer agents, which could help chemists to develop an effective and safe anticancer drug. It also highlighted the importance of the extraction and sampling timing as a major factor in the biochemical composition yield of *Cystoseira,* which should be carefully considered when investigating the bioactivity of the algae-derived bioactive substances.

Given that *Cystoseira* spp. have shown to contain both primary and secondary bioactive compounds abundantly and taking into account that *Cystoseira* has shown a potent cytotoxic activity, we assumed that those bioactive compounds have more bioactivities than have been described in chapter 3. Therefore, it was interesting to continue investigating *Cystoseira* extracts for anticancer activity via immunomodulation as we aimed to test our *Cystoseira*

extracts for stimulating the immune cells to produce cytokines that can fight cancer. However, surprisingly the extracts showed exciting results on the inhibition of TNF-α and IL-1β release. We then decided next to investigate the anti-inflammation activity of *Cystoseira* spp. extracts. Considering that the methanol extracts of *C. tamariscifolia, C. crinita* and *C. compressa* was the most biologically active in cytotoxicity on the cancer cell lines; therefore, it was chosen for the anti-proinflammatory study. The pro-inflammatory cytokines TNF-α and IL-1β are mostly secreted in the inflammatory condition and perform as messengers, which promote the inflammatory process (Jung *et al.,* 2009). Thus, the production downregulation of the pro-inflammatory cytokine is one of the highly crucial approaches in anti-inflammatory therapy. In this study, therefore, the crude methanol extract of the three *Cystoseira* species was employed to suppress the release of TNF-α and IL-1β from stimulated M1 macrophage-like cells. These macrophage-like cells were obtained by activating the human leukaemia cell line THP-1 using PMA at a concentration of 500 ng mL^{-1}. This method has shown to be reliable and has been conducted in a number of immunomodulation studies (Foey & Crean, 2013; Al-Shabany *et al.,* 2016). The macrophages were then stimulated using LPA and LTA and live and heat-killed *E. coli* for two hours. Overall, our results indicate the release of pro-inflammatory cytokines TNF-α and IL-1β from M1 macrophage-like cells was significantly reduced by *C. tamariscifolia, C. crinita* crude extracts (see figure 4.1). Similar observations were reported in studies on extracts from brown seaweeds *Fucus evanescens, Sargassum hemiphyllum* and *Ecklonia* sp. in which extracts reduce the elevated levels of pro-inflammatory cytokines TNF-α and IL-1 in various LPS-stimulated immune cells (Kuznetsova, 2009; Yang *et al.,* 2016; Hwang *et al.,* 2011). In the contest of *Cystoseira*, our result demonstrated higher inhibition of the release of the pro-inflammatory cytokine TNF-α in LPS-stimulated THP-1 human macrophages than that in *C. usneoides* extract reported by De Los Reyes *et al.* (2013). Despite that, it has shown potent anti-proinflammatory activity in a study by Mhadhebi *et al*. (2014); in this study, *C. compressa* showed the lowest anti-proinflammatory activity. During the process of washing the *C. compressa* samples, a large amount of dark brown fluid was seen coming out of the samples, and the fluid continued to come out even during the drying process. The author speculates that this may lead to losing an important level of bioactive components, leading to a low level of anti-inflammatory activity.

The fact that our *Cystoseira* extracts significantly reduced the release of both TNF-α and IL-1β suggests the possibility that the extracts have an anti-multi cytokines effect that might be used against great inflammatory activities in tissues for therapeutic purposes. This could

include cancer therapy as TNF-α and IL-1β have been consistently linked with tumorigenesis and promoting tumours (Zbakh *et al*., 2020). They enhance mutagenesis, uncontrolled cell proliferation, angiogenesis, invasion and may also limit cancer cell apoptosis (Niccolai *et al*., 2020). TNF, particularly in middle and elderly age, can promote the proliferation and cell survival of some malignant cell lines by activating the antiapoptotic pathways hinging upon the activity of NFkB and protein kinase family members (Mocellin & Nitti, 2008; Balkwill, 2006). IL-1β signalling also contributes to tumour growth through activation of NF-κB and contribute to infiltrating Myeloid-Derived Suppressor Cells (MDSCs) and tumour-associated macrophages (TAMs), which contribute to the deactivation of cytotoxic T cells in the tumour site (Kaplanov *et al*., 2019: Rébé & Ghiringhelli, 2020). This involvement in pathological processes malignant disease suggests that TNF-α and IL-1β may be a target for cancer therapy by *Cystoseira* spp. The extracts could block a certain TNF-α and IL-1β signalling pathway either by affecting the functional activity of the NF-κB or by blocking the function of an accessory protein necessary for NF-κB activation. The fact that the extracts could inhibit cancer-supporting cytokines and demonstrated strong cytotoxic activity suggests that these extracts may be beneficial in tumours therapy by performing double actions via both the inhibition of the release of TNF-α and IL-1β and the killing of cancer cells.

Given that *C. tamariscifolia* and *C. crinita* methanol extracts had the highest cytokines release inhabitation effect without showing cytotoxicity impact; therefore, they were chosen for further work. Nevertheless, it is still possible that the extract anti-proinflammatory activity could happen due to interaction between the LPS and endotoxin from the complex seaweed structures (Yermak *et al.,* 2016; Yermak *et al.,* 2020). Moreover, the interactions between transport conditions, storage of samples, extraction medium, storage of extracts, filter type could also lead to extracts contamination (Spaan *et al.,* 2007). For these reasons, a LAL endotoxin assay was performed to rule out the possibility that bacterial endotoxins or LPS-like compounds contaminations might contribute to observed effects. Here we show that all extracts and experiments preparations used for the *in vitro* studies were free of endotoxin, but it also showed that the extracts had an anti-endotoxin activity which was previously reported by (Kuznetsova *et al.,* 2014). However, the extracts did not completely block the endotoxin LPS; therefore, further experiments were performed to clear that weather the inhibition of TNF-α release was due to blocking LPS from stimulating M1- like cells. The extracts were added to media and cells both after and with adding the LPS at the same time. A similar anti-proinflammatory activity was observed in both cases, which suggests that a combination of both the blocking a

particular pathway and anti-endotoxin activities cussed the inhibition of the TNF-α release from M1 macrophage-like cells. These observations suggest that *Cystoseira* spp is a valuable source of natural anti-inflammatory products and thus offer a novel approach that might help in immune disease therapy.

The anti-inflammatory natural products discovery presents an opportunity to improve the management of inflammatory diseases. In fact, investigating physiological action, chemical characterisation, purification and identification involving the usage of potential natural anti-inflammatory products allows specialists to employ these constituents safely and effectively as anti-inflammatory drugs (Fürst & Zündorf, 2015). Indeed, the anti-inflammatory active compounds have been isolated from brown seaweeds and serve as drugs or as a model for preparing drugs (Khan *et al.,* 2007; Kim *et al.,* 2010; Vo *et al.,* 2011). However, to the best of our knowledge, the identification of compounds that demonstrated anti-proinflammatory activity from brown seaweeds *Cystoseira crinita* have not been investigated before. Therefore, this study was conducted to understand further and discover and identify the potential of *Cystoseira*-derived compounds with anti-inflammatory activity *In vitro*. Multiple techniques may be used in order to obtain fraction and isolate components from the natural source, for example, determination of the purity of an isolated substance or the concentration of a single or group of substances in a mixture by fingerprinting (Kim *et al.,* 2011; Uliyanchenko, 2017). Profiling and analysing of the component take place in column chromatography by a different affinity between the stationary and mobile phases based on the difference in solubility at the given temperature (Lee *et al.,* 2014). Moreover, solubility, shape, size, electrical charge and several other features may influence in various ways in which each group of compounds (Wolfender *et al.,* 2015).

In chapter (5), we aimed to chromatographic profiling of the active anti-proinflammatory as an initial step for isolating and detecting the responsible compound for the inhibition of cytokines release from stimulated M1 macrophage-like cells. Methanol extracts of *C. tamariscifolia* and *C. crinita* showed a similar chromatographic profile with several sharp and well-separated peaks recorded at 270-280 nm, as shown in figure (5.1). The well separating of peaks was an important factor in deciding to purify and analyse the active components. Therefore, we performed a fractionation process by HPLC following the same profiling method in order to purify the anti-proinflammatory compounds, which has been used to purify anti-inflammatory agents from brown seaweeds in several studies (Kim *et al.,* 2009; Lee *et al.,* 2015; Chakraborty and Dhara, 2019). As a result of the initial fractionation, six HPLC fractions have been obtained

from each Methanol extract of *C. tamariscifolia* and *C. crinita*. The fractions were concentrated and then examined for their TNF-α release from LPS- activated M1 macrophage-like cells. Both T5 and T6 fractions from *C. tamariscifolia* as well as fractions C1 and C3 from *C. crinita* (figure5.2) showed a decrease in the expression of TNF-α in LPS-stimulated M1macrophage-like cells. A similar observation was reported by Yoon *et al.* (2009), who tested the inhibition effects of *Dictyota. dichotoma* fractions on TNF-α, release in LPS-stimulated RAW 264.7 macrophage cells. They confirmed that methanol and chloroform fractions of *Dictyota. dichotoma* also showed decreases in the expression of TNF-α. The active compounds are usually discovered by several cycles of fractionation of the extract linked with testing of each fraction until the pure compounds are isolated from the crude extracts (Atanasov *et al.,* 2015). In this case, therefore, we chose C1 in which showed significant inhibition of the TNF-α was sup-separated into three sup-fractions C1a, C1b and C1c using HPLC (figure 5.4) with the same method of the initial fractionation as described in chapter 2. We were able to reach a concentration of approximately 250 µg mL^{-1} of the fractions by repeating the sup-separating process and then evaporating. As shown in figure (5.5), our pre-treatment experiments results showed that the C1c had pure anti-TNF-α release compounds. In fact, the C1c fraction inhibited the TNF-α significantly at 100 µg mL^{-1} which is higher than the effect of 250 µg mL^{-1} C1 fraction. The data suggest that the existence of other compounds in the extracts could slightly interfere with the activity of the anti-TNF-α bioactive compounds (Recio *et al.,* 2012; Nunes *et al.,* 2020). This also suggests that our purification cycles process was able to isolate the anti-inflammatory compounds inclusively.

Despite that, there are various chromatography and spectrometry techniques in which have been used for the identification of individual bioactive compounds from various natural sources (see chapter 1), the qualitative analysis using high-performance liquid chromatography (HPLC) in combination with mass spectrometry (MS) still, the most successful method in the identification of natural products (Kumar, 2017). Among all studies reported in the general introduction chapter on brown algal LC-MS analysis, only a few of them analysed the presence of individual anti-proinflammatory compounds from brown seaweeds. Also, as long as the author is aware, no study has conducted the identification of anti-TNF-α release from *C. crinita*. Thus, this study was an attempt to identify anti-TNF-α release active compounds in the purified fraction from *C. crinita*. Here the LC-UV analysis showed that the peaks of interest were observed at the solvent front at about 2 mins with only a very slight and broad upward wave of absorption after 10 mins and a small peak at about 39mins (see figure 5.6). These

peaks did not appear in the subsequent analyses of the C1 fraction due to the high concentration of the C1 fraction used for the fractionation process. This could be explained by the very sharp and big peaks that appeared in the initial HPLC profiling (figure 5.1b) as the concentration of the sample injected was less than 100 µg mL^{-1} which is similar to the concentration used for the LC-UV analysis.

Usually, spectrometry studies conducted in the identification of bioactive substances from brown seaweeds by means of mass spectrometry require either a positive or negative modulus (Rajauria *et al.,* 2016; Steevensz *et al.,* 2012; Wang *et al.,* 2016). However, in the present study, the use of both positive modules was taken into consideration. Ore results of LC/ESI-MS chromatograms for positive and negative ion modes showed numerous peaks of interesting compounds. The accurate m/z values of the ions of interest in these peaks were consistent with the presence of mannitol and fatty acids, namely, nonanedioic acid, undecanedioc acid, dihydroxy stearic acid, palmitic acid and oleic acid (figures 5.8 a,b,c,d and e). Similar compounds have been previously identified in extracts from *Cystoseira* species, including *C. crinita, C barbata*, *C. nodicaulis*, *C. tamariscifolia*, *C. usneoides, C. abies-marina, C. humilis* and *C. osmundacea* (Amico *et al.,* 1976; Usov & Chizhov, 1993; Chizhov *et al.,* 1998; Ivanova *et al.,* 2013; Duarte, 2016). In fact, in the aspect of the anti-inflammatory activity and in line with our observations, mannitol and fatty acids have been reported to have anti-NTF-α release effect (Schreibman *et al.,* 2018; Infante *et al.,* 2012; Wang & Huang, 2015; Li *et al.,* 2019; Calder 2006; Fontaine-Bisson *et al.,* 2007). However, in other studies, mannitol has been shown to have a pro-inflammatory effect, as it has shown to increase in TNF-α release from LPS-Stimulated feline PBMC (Morohoshi *et al.,* 1996; Haak & DeClue, 2008). We suggest that the impact of the C1c from *C. crinita* in the inhibition of TNF-α in stimulated M1macrophage-like cells may be a combination of both mannitol and fatty acids present in the *C. crinita* methanol extract, and they should account for the observed anti-inflammatory activities. However, the mechanism of the inhibition of TNF-α in stimulated M1macrophage-like cells is not yet fully understood. Therefore, further study is necessary to address how mannitol and fatty acids from the *Cystoseira* extract are able to block TNF-α release in stimulated macrophages.

In conclusion, the present study highlighted the anticancer and anti-inflammatory activity of crude extracts of *Cystoseira* spp. According to the results *Cystoseira* spp. have high level of primary and secondary bioactive compounds. The study also confirmed that the three *Cystoseira* spices demonstrated potent anticancer activity against human leukaemia HL60 and

THP-1, prostate PC3 and lymphoma REC-1 cancer cell lines and both biochemical composition and cytotoxicity in *C. tamariscifolia* are subject to seasonality and extraction methods. Moreover, this study reveals that the methanol extract of *C. tamariscifolia* and *C. crinita* extracts significantly inhibited the release of TNF-α and IL-1β. *C. crinita* methanolic sup-fraction C1c from had a significant anti-TNF-α release effect and LC-MS analysis of the fraction showed that it contains fatty acids and mannitol. These data suggest that sampling times and extraction methods should be considered to maximise the possibility of finding novel bioactive compounds in *Cystoseira*. The study also suggests that *C. crinita* is a valuable source of anti-inflammatory agents. This should provide helpful information for medicinal chemists in their attempts to develop anticancer and immunomodulatory agents. However, more research on this topic is needed to understand *Cystoseira* bioactive compounds mechanisms of action.

6.2 Future work

In recent years, there has been a dynamic increase in the number of discovered natural compounds that belong to various groups of primary and secondary metabolites. Much attention has been paid by the researchers toward natural bioactive compounds from brown seaweeds as functional ingredients in pharmaceutical strategies. The wide ranges of biological activities associated with *Cystoseira*-derived bioactive compounds have the potential to expand its health beneficial value in pharmaceutical industries, and hence, it can be suggested that the *Cystoseira* an alternative source for synthetic ingredients that can contribute to human well-being, by being a part of new functional anticancer and anti-inflammatory agents. Although still in their infancy and there is a paucity of the information reported in the literature, which only contains studies on *in vitro* or animal models studies on the anticancer and anti-inflammatory effects of *Cystoseira* compounds should be corroborated by clinical trials. Human studies could strengthen the choice of *Cystoseira* products as potential bioactive compounds for the therapy of cancer and inflammatory disease. Not many industrial competent products have been developed utilizing these bioactive compounds for the health and well-being of humans. Primarily this is because extensive human trials studies are pre-requisites to establish *Cystoseira* as an excellent raw material for pharmaceutical agents at the commercial level. Therefore, a better knowledge of these *Cystoseira* molecules should be associated with an implementation in the extraction method and seasonality and purification procedures in order to obtain *Cystoseira* extracts with standardized concentrations to be applied in future trials. Indeed, the choice of a proper extraction method and seasonality can deeply influence the presence and concentration of the bioactive compounds. Therefore, further studies need to be designed to explore the bioactivity of these compounds for long-term health beneficial effects. In the current study, bioactive crude extracts from *Cystoseira* spp show promise for use in functional pharmaceutical products for anticancer therapies (Chapter 3). There were, however, some limitations in this study, including cost and time, which prevent us from identifying the active cytotoxic compounds presented in the extracts. As many *Cystoseira* potential cytotoxic compounds are favourable to have an effect on human cancer cell lines, a number of researchers are looking for purifying and then identifying brown seaweeds compounds that can show cytotoxicity effect on cancer cells (Mhadhebi *et al.,* 2014; Taskin *et al.,* 2010; Zbakh *et al.,* 2020; Moussa *et al.,* 2020). In line with these studies, therefore, the future work direction should be purifying and identifying *Cystoseira* active cytotoxic compounds. It can start with several cycles of purifications using HPLC and testing the

cytotoxic effect of the purified fractions on cancer cell lines, and then different identification strategies can be applied. One strategy is identifying the anticancer compounds in the active fractions using a positive and negative modulus of an LC-MS method. The technique has been previously employed to identify bioactive compounds extracted from brown seaweeds (Liu *et al.,* 2012; Belda *et al* ., 2016; Rupérez *et al.,* 2002; Pereira *et al.,* 2017). It is still the most successful method in the identification of natural products (Kumar, 2017). LC-MS strategy showed beneficial effects as it identified anti-proinflammatory compounds in this study which suggest it might be useful for identifying the cytotoxic compounds in *Cystoseira* extracts. Other strategies include using the centrifugal partition chromatography (CPC) technique has also been used for the purification of molecules, separation of compounds, fractionation of crude extracts. CPC is a liquid-liquid chromatography technique that relies on two immiscible liquid phases and does not require a solid phase. The two liquids work as mobile and stationary phases, which replaces the silica-based column in flash chromatography and HPLC. The centrifugal field generates the stationary phase in the column by rotation (Kim *et al.,* 2011). Here, the compounds separation process is based on the difference in the distribution of components in two immiscible liquid phases. This enables the isolation and purification of large quantities of compounds with purities of greater than 90% in a one-step process (Lee *et al.,* 2014). LC-MS then can also be applied to identify the isolated active compounds by CPC.

In our study, *Cystoseira* crude extracts exhibited relatively high cytotoxicity on cancer cell lines *in vitro*. Despite there are shreds of evidence that *Cystoseira* extracts are toxic to the cancer cells while having no harmful effect *in vivo* and *in vitro* toxicity on normal cells of the body part in which cancer had developed (Zubía *et al.,* 2020; Güner *et al.,* 2015). It is still possible that these extracts could have other undesirable side effects, or they are actually inactive *in vivo*. Therefore, an animal *in vivo* trial should be conducted in future work to evaluate the cytotoxic effect on cancer cells where cancer developed in the body. One strategy is using Albino mice for *in vivo* experiments with taking into consideration the ethical guidelines for the investigation of experimental pain in conscious animals. Oral administration of seaweed powder dose (15,000-2,000 mg kg^{-1} body weight) for 28 days can be conducted under regulations from the Environmental Protection Agency (EPA) and the Organization for Economic Cooperation and Development (OECD). An observation of the mice clinical signs and microscopic tissue examination, as well as the median lethal dose (LD50) assay, should give better knowledge about the safety and the efficiency of the *Cystoseira* anticancer extracts and compounds.

This thesis serves as a baseline for anti-proinflammatory data of extracts and purified fractions from *Cystoseira* spp. Here, *Cystoseira* spp extracts and purified fractions showed a potent anti-proinflammatory activity by inhibiting cytokines TNF-α and IL-1β release from stimulated M1 macrophage-like cells. The active purified fraction then was found to contain mannitol and fatty acids. However, it was not clear how the compounds blocked the cytokines in particular TNF-α release; hence, further investigations are required in future work to reveal their exact mode of action. In previous studies, fatty acids have been shown to inhibit LPS-induced TNF-α secretion in both *in vitro* and *in vivo* in several direct anti-inflammatory mechanisms (de Lima-Salgado *et al.,* 2011; Wang & Huang, 2015; Li *et al.,* 2019; Calder, 2006; Fontaine-Bisson *et al.,* 2007). However, most likely fatty acids reduce the release of TNF-α in LPS stimulated immune cells by preventing activation of NF-κB (Tzeng *et al.,* 2019; Kawa *et al.,* 2019). NF-κB is a key transcription factor of M1 macrophages and is required for the induction of a large number of inflammatory genes, including those encoding TNF-α (Weldon *et al.,* 2007;). One possible pathway is that bacterial LPS binding to TLR4, leading to the activation of NF-κB signalling, which in the nucleus promotes the transcription of NF-κB-dependent genes, such as NLRP3, Pro-IL-1β and Pro-IL-18. Those are necessary for the induction of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, in macrophages (Yao *et al.,* 2009; Davignon *et al.,* 2013). These cytokines subsequently are able again to activate NF-κB in innate immune cells, thus inducing the expression of additional inflammatory cytokines, including TNF-α, leading to further promotion of inflammation (Liu *et al.,* 2017). Also, the activation of NF-κB consequently induces TNF-α, which lead to an increase of PGE2 release, and so more stimulation of NF-κB by PGE2 (Calder, 2006). Given that NF-κB appears to be a key factor in mediating most inflammation mechanisms, the inhibition of NF-κB may be the crucial point for the effects of the fatty acids on TNF-α release and inflammation. Therefore, future work directions should target the NF-κB signalling pathway to evaluate the mechanism of the anti-proinflammatory compounds in *Cystoseira* extract.

In order to investigate whether the suppressive effect of *Cystoseira* active compounds on TNF-α release from macrophages was due to inhibition of NF-κB expression, the activation level of the NF-κB promoter could be measured by monitoring its luciferase activity in stimulated macrophages (Jang *et al.,* 2020). ELISA and immunofluorescent staining *in vitro* can also be used for the evaluation of NF-κB activation (Su *et al.,* 2019). The level of the NF-κB could also be facilitated by examining the nuclear translocation patterns p65 and p50, which are two subunits composing NF-κB and analysed by western blotting using nuclear lysates of macrophage cells. The level of p50 and p65 protein detected in the treated LPS stimulated

macrophage cells indicates the level of NF-κB activation. This should give a better idea about how the extracts may inhibit the NF-κB signalling pathway and therefore reduced the TNF-α release in macrophages.

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Appendices

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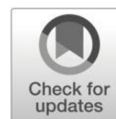
23RD INTERNATIONAL SEAWEED SYMPOSIUM, JEJU



The cytotoxic activity of extracts of the brown alga *Cystoseira tamariscifolia* (Hudson) Papenfuss, against cancer cell lines changes seasonally

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Abstract

Brown seaweeds have been highlighted for their abundant production of bioactive substances that may provide novel drugs or drug scaffolds for a range of diseases, particularly cancer. Indeed, a number of promising compounds that can modulate growth arrest or apoptosis have already been isolated. As previous work has highlighted seasonal differences in concentrations of secondary metabolites, this study aimed to evaluate seasonal variation in the cytotoxic anticancer activity of *Cystoseira tamariscifolia* extracts. Primary and secondary metabolites were measured using colourimetric techniques and extracts were exposed to human leukaemia, HL60 and THP-1, and human prostate cancer PC3 cell lines in vitro, and cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results confirm a large but inconsistent seasonal variation in the concentrations of biomolecules, although, generally, levels were higher in spring and summer. IC_{50} values for cytotoxicity also showed variability both seasonally and with extraction protocol, but again, this was inconsistent. These data suggest that when collecting brown algae in order to isolate novel bioactive compounds, repeated sampling across annual or biotic cycles of the seaweed and the use of multiple solvent extraction methods could give a greater possibility of discovering bioactive lead compounds.

Keywords Anticancer · Chemical metabolites · Seasonal variation · Seaweed · Phaeophyta

Introduction

Cancer has become one of the major causes of premature death in an ageing population. With the risk of developing cancer approaching 50% by the age of 80, combined with current life expectancy figures that are increasing in developing countries, cancer is likely to remain a major affliction to patients and a challenge to healthcare services. The most common treatments for cancer are still surgery, radiotherapy and chemotherapy, including direct and indirect hormonal and immune therapies (Coffelt and de-Visser 2015; Senthebane et al. 2017). Currently, most of the chemotherapeutic strategies used to treat different cancers are not completely effective and are often associated with severe side effects that can significantly reduce the quality of life of the patient. Thus, much research is being dedicated to finding novel compounds that show clinical efficacy with reduced side effects, with much of this research concentrated on natural products including those from marine algae (Anand et al. 2016). Bioactive compounds from brown seaweeds have been highlighted for their importance as anticancer agents (Ashwini et al. 2016), and most of these compounds are generally safe and are abundant in brown seaweeds, which make them a promising potential source of therapeutic compounds (Zubia et al. 2009). A wide range of these bioactive ingredients has been reported to have anti-tumour activity. Polyphenols have been linked with anticancer activities of seaweed crude extracts, for instance, phloroglucinol and dioxinodehydroeckol from brown algae display an antiproliferative activity against colon and breast tumours (Murphy et al. 2014; Lopes-Costa et al. 2017). Similarly, phloroglucinol from an *Ecklonia cava* extract showed apoptosis-enhancing effects on the MCF-7 human breast cancer cell line (Kong et al. 2009). Furthermore, crude extracts from brown algae *Palmaria palmata*, *Laminaria setchellii* and *Macrocystis integrifolia* exhibited cytotoxicity against the cervical cancer HeLa cell line (Yuan and Walsh 2006). The anticancer

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Anticancer and immunomodulatory activities of the brown seaweeds *Cystoseira* spp.

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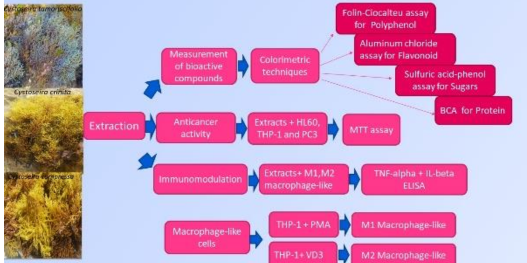
Introduction

According to the American Cancer Society, the global cancer burden is expected to grow to 27 million new cases/year and by 2050, there will be 17.5 million cancer deaths/year. Finding new anticancer and immunomodulatory agents is very important for the treatment of cancer, and the brown marine algae are a valuable source for developing novel agents for clinical application (1). Therefore, the isolation of novel anticancer components from the brown seaweed *Cystoseira* and a study of their mechanism of action is very attractive to assess their potential as a poorly explored source for pharmacological applications (2). This study reveals the bioactive components of three *Cystoseira* species, together with their antitumor and immunomodulatory potential as direct and indirect cancer treatments according to their influence on the viability of cancer cell lines and the cytokine production by macrophage-like cells. This should provide useful information for medicinal chemists in their attempts to develop potent anticancer and immunomodulatory agents.

Methods



Results

To asses the bioactive compounds *of Cystoseira tamariscifolia, C. crinita* and *C. compressa* from the U.K. and Libya, four extraction solvents were applied to material, and the concentrations of primary and secondary metabolites measured. Methanol (100%) was generally the most effective solvent for extracting all compounds, although all solvents gave detectable levels of all the investigated metabolites, the exception being chloroform that interfered with the BCA assay for protein.

The concentrations of bioactive compound compositions of the brown seaweed *Cystoseira* spp. collected from the U.K. and Libyan coasts.

Chemical component ($mg\ g^{-1}$ DW)	*Cystoseira* species	Extracts			
		100%MeOH	70%MeOH	Water	Chloroform
Polyphenols	*tamariscifolia*	102.23 ± 1.85	57.70 ± 2.06	83.24 ± 1.03	41.99 ± 0.90
	crinita	153.03 ± 15.1	95.68 ± 6.28	102 ± 11.38	26.86 ± 4.94
	compressa	58.70 ± 0.62	74.71 ± 6.12	41.98 ± 8.29	33.35 ± 3.97
Flavonoids	*tamariscifolia*	22.87 ± 0.80	5.55 ± 0.46	8.56 ± 0.71	22.27 ± 0.66
	crinita	57.51 ± 3.88	35.55 ± 5.54	11.25 ± 2.02	47.22 ± 2.28
	compressa	10.22 ± 0.72	10.67 ± 1.18	6.21 ± 0.96	14.43 ± 0.25
Polysaccharides	*Tamariscifolia*	48.84 ± 3.66	8.16 ± 0.40	14.95 ± 1.85	42.84 ± 3.84
	crinita	47.85 ± 6.17	18.35 ± 3.62	20.28 ± 4.17	17.47 ± 1.05
	compressa	0.73 ± 0.17	2.31 ± 0.88	5.06 ± 0.31	1.95 ± 0.35
Proteins	*tamariscifolia*	9.28 ± 0.28	8.10 ± 0.59	7.18 ± 0.63	-
	crinita	12.63 ± 0.71	6.96 ± 0.36	3.93 ± 0.63	-
	compressa	10.50 ± 0.19	6.73 ± 0.01	6.37 ± 0.29	-

DW: dry weight. Total polyphenol content: mg phloroglucinol acid equivalents g^{-1} DW; Total flavonoid content: mg quercetin equivalents g^{-1} DW; Total polysaccharide: mg glucose equivalents g^{-1} DW ; Total protein: mg albumin bovine serum equivalents g^{-1} DW. Values are presented as mean ± SD (n = 3).

Results (Continued)

Three different cell lines, HL-60, THP-1 and PC3 cells, representing two leukaemia-derived lines and a prostate cancer line, respectively were treated with increasing concentrations of C. *tamariscifolia*, C. *crinita* and C. *compressa* extracts and cell vitality was assessed via the MTT assay. Cells were separately treated with crude extracts from each of the four solvents. Significant anticancer activity was found with IC_{50} = 10.739 ± 4.21, 64.906 ± 3.69, and 23.031 ± 5.04 µg ml^{-1} for HL60, THP-1 and PC3 cell lines, respectively.

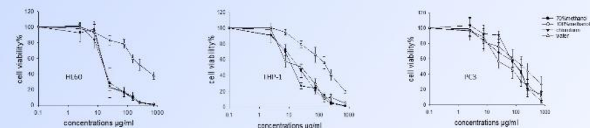


Figure 1. Percentage of cell viability in comparison to control (mean ± SD; n = 9). HL60, THP-1 and PC3 cell lines were exposed for 72 h to crude extracts of the brown alga *Cystoseira tamariscifolia*.

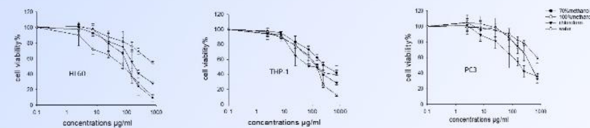


Figure 2. Percentage of cell viability in comparison to control (mean ± SD; n = 9). HL60, THP-1 and PC3 cell lines were exposed for 72 h to crude extracts of the brown alga *Cystoseira crinita*.

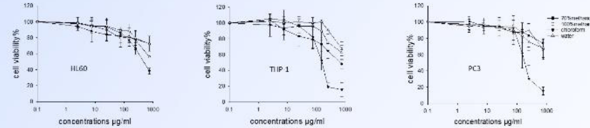


Figure 3. Percentage of cell viability in comparison to control (mean ± SD; n = 9). HL60, THP-1 and PC3 cell lines were exposed for 72 h to crude extracts of the brown alga *Cystoseira compressa*.

Since extracts of *Cystoseira* potently inhibited the viability of the cancer cells and did not kill the macrophage-like cells, we continued to investigate whether they suppressed the production of pro-inflammatory cytokines such as TNF-α and IL-β in LPS stimulated macrophage-like cells. After 18-h incubation with both LPS (1 µg mL^{-1}) and crude extracts of the seaweeds (25, 75, 150, or 250 µg mL^{-1}), a significant inhibition of TNF-α and IL-β release was noted.

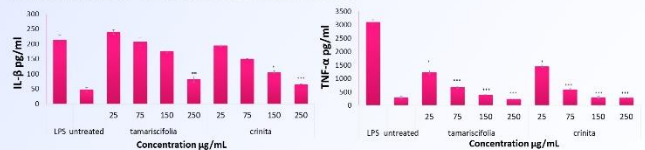


Figure 4. Effect of *Cystoseira* extracts on TNF-α and IL- β production in LPS-stimulated macrophage-like cells. TNF-α and IL- β produced and released into the culture medium was assayed by the ELISA method. The data represent the mean ± SD of triplicate experiments. * P<0.05 vs. LPS alone.

Conclusion

Our results indicate that bioactive compounds extracted from *Cystoseira tamariscifolia, C. critina* and *C. compressa* have potent anticancer and immunomodulatory activities. The study shows that the three species of *Cystoseira* demonstrated potent anti-cancer activity against human leukaemia and prostate cell lines and that *C. tamariscifolia* and *C. crinita* significantly inhibited the production of TNF-α and IL- β in comparison with the LPS control. Further research is underway to identify and purify the bioactive compounds in the extracts that are responsible for the observed anticancer activity and to understand their modes of action.

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