

Phlorotannin and its Derivatives, a Potential Antiviral Molecule from Brown Seaweeds, an Overview¹

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Abstract—Research on seaweeds provides a continual discovery of natural bioactive compounds. The review presents new information on studies of the potential and specific antiviral action of phlorotannin and their derivatives from marine brown algae. Phlorotannin is a polyphenolic derivative and a secondary metabolite from marine brown algae which exhibits a high quality of biological properties. Phlorotannin has a variety of biological activities that include antioxidant, anticancer, antiviral, anti-diabetic, anti-allergic, antibacterial, antihypertensive and immune modulating activities. These phlorotannin properties were revealed by various biochemical and cell-based assays in vitro. This distinctive polyphenol from the marine brown algae may be a potential pharmaceutical and nutraceutical compound. In this review, the extraction, quantification, characterization, purification, and biological applications of phlorotannin are discussed, and antiviral potential is described in detail.

Keywords: Marine polyphenols, phlorotannin, brown algae, antiviral, pharmaceutical, nutraceutical activities

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INTRODUCTION

The mankind has always been faced by a lot of threats throughout history by numerous pathogens like viruses, bacteria, fungus, and certain biological disorders due to mutations, cancer, immune deficiency, diabetes etc. Recently, frequent outbreaks of several deadly infectious diseases are occurring, which lack either proper treatment or preventive remedies, as the pathogens became resistant to existing approved drugs, which may also exhibit side effects to some extent. This review article explores the chances of prevention and treatment against pathogens and disorders through naturally available phlorotannin extracted from marine algal resources. Seaweeds have been identified as an abundant and competent natural source of biologically active compounds owing to their variety of biological properties to combat various harmful infections including viruses. Seaweeds are being utilized as a part of dietary and medicinal supplements for a prolonged period of time [75]. Seaweeds exhibit an outstanding source of bioactive metabolites that could constitute functional leads in the development of new pharmaceutical molecules [10]. The marine algal species are one of the crucial sources of commercially predominant metabolites

mainly polysaccharides and polyphenols [39]. Among numerous classes of algae, the brown algal family *Phaeophyceae* is prominent for acquiring phenolic compounds [62]. Most of the polyphenols revealed and explored from marine resources are of macroalgal origin [97]. The crucial categories of phenols include phlorotannins, terpenoids, phenolic pigments, and bromophenols, which are mostly available in marine brown algae.

Phlorotannin is the phloroglucinol polymer (1,3,5-trihydroxybenzene), which is the mostly investigated group among the phenolic compounds [84]. Because of a wide range of bioactive resources, substantial recognition has been drawn lately to phlorotannin [34]. Phlorotannin comprises about 25% of the dry weight of brown algae. Phlorotannin is the phenolic metabolite that is bound to the subcellular regions of brown algal species. Similar to the tannins of terrestrial plants, the phenolic compounds from seaweeds possess a major composition of hydroxyl groups (–OH), which are mostly water soluble, firmly attached to proteins, polysaccharides, and biopolymers. Phlorotannin has an ability to chelate divalent metals [57, 62, 95]. Phlorotannin have an extensive mass range (from 1 to 650 kDa and higher) with an unique polymeric structure and often exhibit in the molecular range between 10–100 kDa [85]. The important role of the

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phlorotannin phenolic compound is to contribute protection against photo oxidative stress influenced by UV b radiation and exhibit the inhibitory effects on melanogenesis [88]. The polyphenols from marine algae are the chemically categorized metabolites comprising several hydroxylated aromatic rings [58].

Phlorotannin possesses a wide range of bioactivity depending on the structure and polymerization [39]. Green and red marine algae carry only a low concentration of phenols, whereas brown marine algae are specifically abundant in phlorotannins with 1% to 14% dry algal biomass. The polyphenolic concentration in the marine brown algae varies according to the season and climatic condition [39]. Phlorotannins are subclassified as phloroglucinol, eckol, phlorofucofuroeckol A, phlorofucofuroeckol B, 2-phloro-eckol, dieckol, 6,6-bieckol, and 8,8-bieckol that have been isolated and characterized [97]. Phlorotannin is present in and extracted particularly from various marine brown algal species, such as *Sargassaceae*, *Alariaceae* and *Fucaceae*. The biologically active phlorotannins have been identified from the brown algae, such as *Ecklonia cava*, *Fucus vesiculosus*, and *Ascophyllum nodosum* [39]. In this literature review, biological activities, mainly antiviral and immune modulating activities of marine algal phlorotannin are discussed as a potential natural drug candidate with their protective outcome in biological systems and human health.

SOURCES OF PHLOROTANNIN

Around 1800 species of marine brown algae were identified, which contain an elevated amount of polyphenols, especially phlorotannins [36]. The phlorotannins from *Fucaceae*, a family of brown seaweeds, were known for their significant contribution to biological activities [15]. Phlorotannin content may oscillate depending on the habitat and vary within the same algal body and population [20]. *Ecklonia cava*, an edible brown seaweed, was explored for various types of phlorotannins, depending on the structure. Shibata et al. [83] reported the phlorotannin contents in *Ecklonia cava* as a crude phlorotannin (3.0%), Phloroglucinol (4.7%), Eckol (6.4%), Fucodiphloroethol G, Phlorofucofuroeckol A (16.6%), Dieckol (22.2%), and 8,8'-bieckol (12.4%). Cuong et al. [18] reported that the phlorotannins found in *Ecklonia cava* included Dieckol (1.52 mg/g dry weight) and Phlorofucofuroeckol A (0.93 mg/g dry weight). Kim et al. [47] reported the total phlorotannin content—3.39 mg PGE/mL—in crude phlorotannin extract from *Ecklonia cava*. *Ecklonia bicyclis* was also investigated for phlorotannin content; the crude extract of phlorotannins (3.1%) included: phloroglucinol (0.9%), phloroglucinol tetramer (4.4%), eckol (7.5%), phlorofucofuroeckol A (21.9%), dieckol (23.4%), and 8,8'-bieckol (24.6%) [83].

Ecklonia kurome contains 3.0% of crude phlorotannins, 2.6% of phloroglucinol, 0.3% of phlorogluci-

nol tetramer, 9.2% of eckol, 28.6% of phlorofucofuroeckol A, and 24.6% of dieckol. *Ecklonia kurome* was found to have 7.8% of 8,8'-bieckol [83]. *Ecklonia stolonifera* was reported to have dieckol (1.52 mg/g dry weight) and Phlorofucofuroeckol A (approx. 1.20 mg/g dry weight) [18]. Heffernan [36] reported the phlorotannin contents from various brown seaweeds: *Fucus serratus* 180.55 (16.98) µg PGE/mg sample, *Himanthalia elongate* 198.28 (9.17) µg PGE/mg sample, and *Cystoseira nodicaulis* 89.14 (2.57) g phloroglucinol equivalents (PGE)/mg sample respectively. Creis et al. [20] reported *Fucus vesiculosus* for its total phlorotannin content ranging from 12 to 23 mg/g dry weight. These species are the main sources of phlorotannins present in marine brown algae, which can be further explored for their therapeutical and nutraceutical potentials.

STRUCTURE AND CLASSIFICATION OF PHLOROTANNIN

The phenolic compounds present in seaweeds include flavonoids, terpenoids, phlorotannins, bromophenols and mycosporine as amino acids. Phlorotannin of brown algae is the only major polyphenol that have been reported [19]. The polyphenols found mostly in marine brown algae are termed as phlorotannins and are scarcely found in red and green algae. The red and green algae are known to have higher concentrations of phenolic acids and flavonoids [55], which comprise about 14% of dry weight of the algae. The phlorotannins are the chief polyphenol present in brown seaweeds and are produced via the acetate-malonate (polyketide) pathway [22]. According to Hakkim et al. [35], the polyphenols from brown seaweeds are the phloroglucinol (1,3,5-trihydroxybenzene) polymers, which are connected together and are also known as phlorotannins. The phlorotannins that exist in the orders Fucales and Laminariales are the polyphenols that play a major role in protecting the algal species from harsh environmental conditions [48].

The bioactivity of phlorotannin depends on its structure and polymerization based on the arrangement of phloroglucinol polymer. The basic structure of phloroglucinol is given in Figure 1. The structure and concentration of phlorotannins change according to the climate, season, habitat and environment [39]. The structures of phlorotannin are classified based on the linkage of monomeric units into six categories, namely fucols, phloroethol, fucophloroethol, fuhalols, eckol and carmalol [13]. They are often found attached to the cell wall and are water soluble. These are the units of phloroglucinol with different degrees of polymerization, which were isolated and characterized by Yong-Xin Li et al. [97]. According to the linkage of the bonds, they are differentiated as fuhalols and phlorethols having ether linkage, fucols having phenyl linkage, fucophlorethols having ether and phenyl link-

Table 1. Antiviral activity of phlorotannins

Species	Collection	Nature of the samples	Extraction type	Compound	Solvent used	Characterization and purification	Assay/ Method	Targetted virus	Inhibitory effect	Ref
<i>Ecklonia arborea</i>	Escalera Zone, North of Punta China	Fresh samples	Sonicated assisted extraction	Polyphenol-rich extracts	Ethanol	—	MTT assay, syn-cytia reduction assay and qPCR	Measles virus (MeV)	Low toxicity, high antiviral activity against MeV, highest Selectivity Index (SI)	63
<i>Ecklonia cava</i>	Cheongsando Island, Republic of Korea	Fresh samples	Ultra-sonication	Phlorofucofuroeckol A	Methanol	HPLC-qTOFMS	Viral-infected Madin-Darby canine kidney cells	Influenza A	Decreasing neuraminidase protein expression against H1N1 H9N2.	17
<i>Eisenia bicylis</i>	NA	NA	NA	Ethyl acetate fraction, phlorofucofuroeckol-A	Methanol	Sephadex LH-20 and RP-18 open column chromatography	MTT assay and tissue culture infectivity dose 50% (TCID ₅₀)	Murine norovirus (MNV)	Powerful antiviral effect against MNV, anti-MNV potential of the extract	26
<i>Eisenia bicylis</i>	Coastal area of Korea and Japan	Fresh samples	Solid-liquid extraction	Phlorotannin	Ethyl acetate fraction	Column chromatography	Bio-luminescence (SEAP) assay	Human papilloma virus	Anti-viral activity against HPV16PVs and HPV18PVs. Concentration range, 50 µg/mL	46
<i>Sargassum muticum</i>	European Atlantic coasts	—	Enzyme assisted extraction	Phenolic extract	Carbohydrase, neutralase, alkalase, protease	FTIR HPLC	Antiviral and cytotoxicity activity	Herpes simplex virus (HSV)	Inhibition against HSV-1 using African green monkey kidney cells	72
<i>Cystoseira myrica</i> and <i>Ulva lactuca</i>	Hurghada (Red sea), Alexandria Mediterranean Sea	Fresh samples	Aqueous extraction	Crude phenolic extract	Distilled water	TLC, GC-MS, NMR	The MTT assay and neutralization assay for antiviral activity	Hepatitis A virus, Coxsackie B4 virus, herpes simplex virus types-1 & 2	Cytopathic effect (CPE) in Vero cells, inhibition effects on HSV-1 replication	90

Table 1. (Contd.)

Species	Collection	Nature of the samples	Extraction type	Compound	Solvent used	Characterization and purification	Assay/ Method	Targetted virus	Inhibitory effect	Ref
<i>Ecklonia cava</i>	Ulleung Trading Co. (Ulleung, Korea)	Refrigerated sample	Solid-liquid extraction	8,4'-Dieckol	Methanol	MS, NMR	Cytopathic effect analysis, reverse transcriptase assay	Human immunodeficiency virus type-1 (HIV-1)	Possess anti-viral property against HIV-1, inhibition of syncytia formation	28
<i>Ecklonia cava</i>	Jeju Island in Korea	Fresh samples	Solid-liquid extraction	Phlorotannin	Ethanol	HPLC	Simultaneous-treatment assay, in vitro antiviral activity	Porcine epidemic diarrhea virus	Inhibiting viral entry and/or viral replication against PEDV	49
<i>Ecklonia cava</i>	Jeju Island, Korea	Fresh samples	Solid-liquid extraction	Phlorotannin derivatives	Ethanol	—	Cell-free cleavage and cell-based cleavage assays	SARS-CoV	Dieckol had the most potent inhibitory activity against SARS-CoV 3CLpro	70
<i>Ecklonia cava</i>	Sungsanpo, Wando, and Namhaedo coast, Korea	Fresh samples	Solid-liquid extraction	Dieckol, 8,8'-bieckol, 8,4'''-dieckol, and phlorofurodieckol A	Methanol	HPLC	HIV-1 Retroviral Transcriptase (RT) Assay, HIV-1 Protease Assay	Human immunodeficiency virus	Inhibitory effect on HIV-1, reverse transcriptase (RT) and protease	2
<i>Ecklonia cava</i>	Jeju Island, Korea	Fresh samples	Solid-liquid extraction	6,6'-Bieckol	Methanol	NMR techniques	MTT, co-cultivation assay, western blot analysis	Human immunodeficiency virus-1	Inhibition against HIV-1 induced syncytia formation, lytic effects, viral p24 antigen production,	6
<i>Ecklonia cava</i>	Jeju Island, Korea	Fresh samples	Solid-liquid extraction	Phlorotannin derivatives	Ethanol	—	Neuraminidase (NA) inhibition assay	Influenza A virus	Inhibition against Neuraminidase from influenza A virus,	76

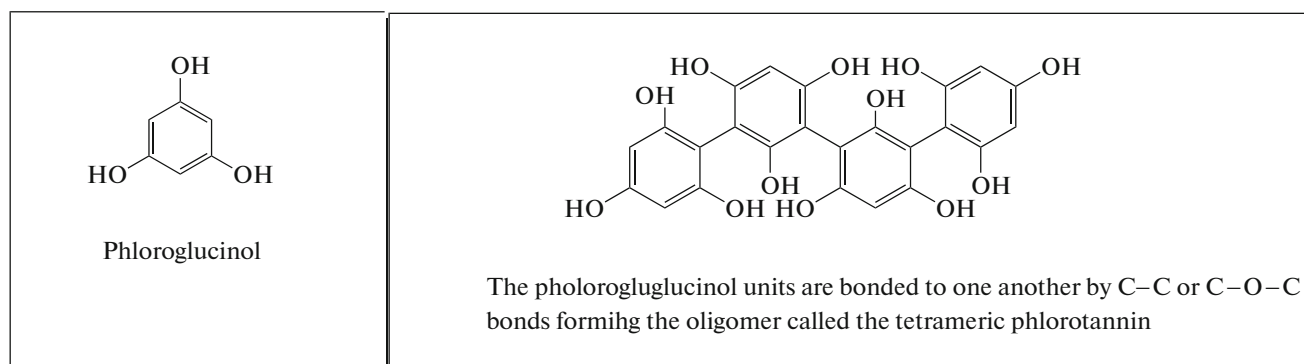


Fig. 1. Basic structure of phlorotannin.

age, and eckol and carmalol having dibenzo-dioxin linkage. These linkages contribute to different structural compositions and, accordingly, to different functions and activity. The coupling is made by C–C and C–O–C bonds of phloroglucinol units [89]. The Classification of phlorotannin structures is given in Fig. 2. Some of the derivatives of phlorotannins reported by Venkatesan et al. [93] include phlorofucofuroeckol A, dieckol, fucodiphlorethol G, 6,6'-bieckol, triphlorethol-A, 2,7'-phloroglucinol-6, and 6'-bieckol.

As the structure of phlorotannin molecules changes, the function also differs. For example, antiviral activity of phlorotannin compounds evaluated by Kwon et al. [49], who reported different activities of various types of phlorotannin, such as phloroglucinol, eckol, 7-phloreckol, phlorofucofuroeckol, and dieckol. Accordingly, phloroglucinol showed no activity and eckol showed maximum activity followed by phlorofucofuroeckol and dieckol. Phlorofucofuroeckol A from *Ecklonia* species, was found to exhibit good antiviral properties [17, 26]. It was shown that each structural composition of phlorotannin has a different effect on the function. The structure of phlorotannin compounds responsible for antiviral activity is given in Fig. 3. Either the addition of OH-groups in the compound or addition of some bonds within the monomers may lead to a structural variation of phlorotannin. These distinctive structures make phlorotannin very unique among other classes of phenolic compounds.

EXTRACTION OF PHLOROTANNIN

Phlorotannin has been isolated from many marine brown algal species and the extraction and identification of various types of phlorotannin is of substantial interest in many industries. Extraction of a particular phlorotannin from brown algae is the most crucial step for exploring their biological significance [41]. Development of the methods for isolation and extraction of phlorotannin becomes significant for examination of the reactivity of phlorotannin. Several methods that

are being used for extracting phlorotannins are as follows below. The primary step of extraction includes washing, drying and grinding of algal samples.

Solvent Extraction

The solid–liquid extraction, liquid–liquid extraction and Soxhlet extraction are the conventional isolation methods of extracting the target compound. The extraction using organic solvents was the most commonly used method. The solvents used include hexane, petroleum ether, cyclohexane, ethanol, methanol, acetone, benzene, dichloromethane, ethyl acetate, and chloroform. Commercially, ethanol plays a major role as a solvent for many extraction procedures owing to its low cost [86]. For extraction of phlorotannins, water-organic solvent mixtures were prominently used; Gall [32] reported the detailed procedures for extraction and purification of phlorotannin using solvents. Maceration is one of the traditional methods of extracting phenolic compounds by immersing the marine algal biomass in a suitable solvent/solvent mixture. For example, to extract phlorotannin from the brown algae *Fucus vesiculosus* and *Ascophyllum nodosum*, the maceration method was applied for 120 min at 50°C with a ratio of 1:5 raw-material:extractant and was followed by spectrophotometry via the Folin–Ciocalteu method [65].

The extraction temperature should be kept below 52°C as higher temperature exposure will degrade the polyphenolic compounds. The maximum yields of polyphenols were obtained using 80% methanol from *Saccharina japonica* (14.9 ± 0.1 mg/g GAE) and 100% methanol from *Undaria pinnatifida* (8.4 ± 0.2 mg/g GAE) [55]. According to the reports, methanol extraction gives the highest recovery of phlorotannins in the organic solvent extraction method [72]. Dieckol from *Ecklonia cava*, *Ecklonia stolonifera* and *Ecklonia bicyclis* showed a good yield—86, 93, and 98%, as compared with other extraction methods [18]. Even though the traditional method provides a higher yield and gives a drawback of usage of a larger amount of

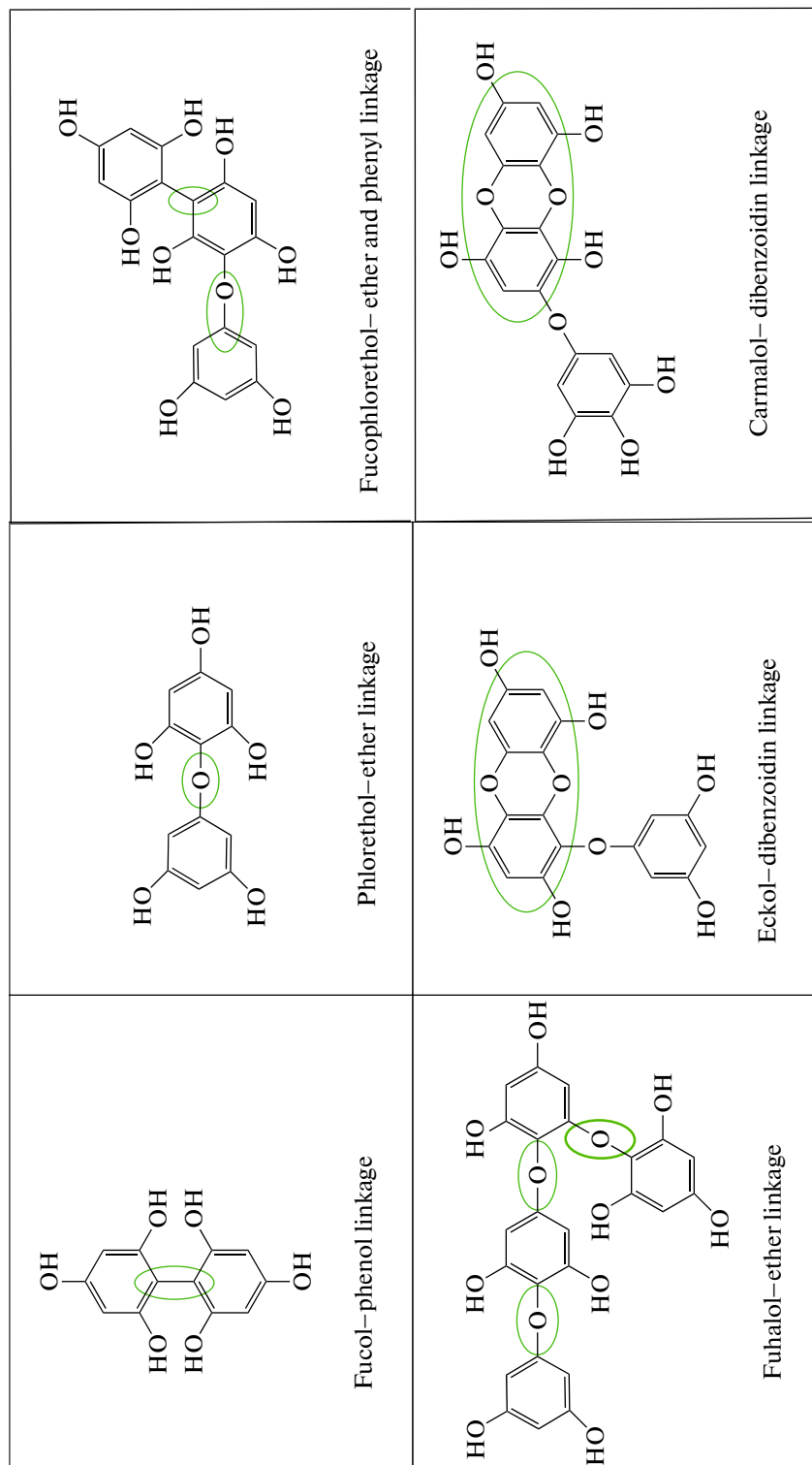


Fig. 2. Structural representation of six different categories of phlorotannin.

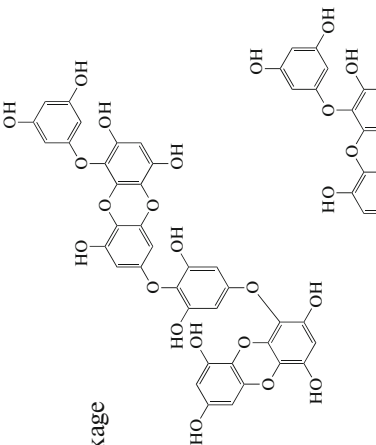
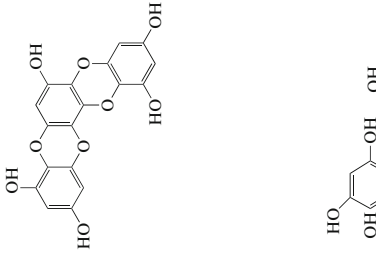
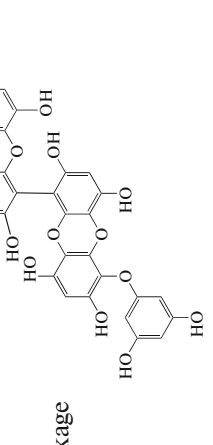
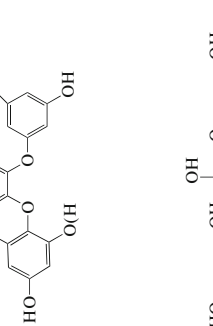
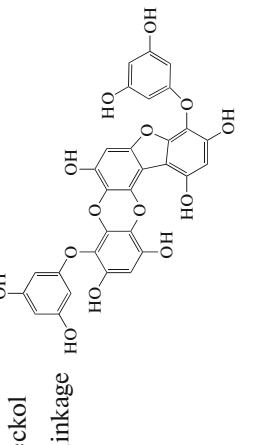
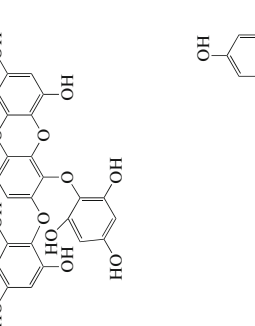
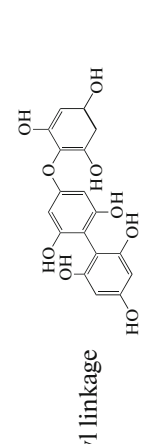
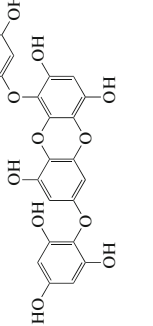
Compound	Structure
<p>Dieckol Dibenzoindin linkage</p> 	<p>Dioxinodehydroeckol Dibenzoindin linkage</p> 
<p>6,6'-Bieckol Dibenzoindin linkage</p> 	<p>Triphlorethol Ether linkage</p> 
<p>Phlorofucofuroeckol A-Dibenzoindin linkage</p> 	<p>2-Phloroeckol Dibenzoindin linkage</p> 
<p>Fucophlorethol Ether and phenyl linkage</p> 	<p>7-Phloroeckol Dibenzoindin linkage</p> 

Fig. 3. Structure of phlorotannin compounds responsible for the antiviral activity.

solvents and longer time duration for complete extraction of phlorotannin compounds.

The major advantage of the Soxhlet extraction method is that it is a continual process in which the solvent can be reused. The time duration is reduced for this type of extraction, it takes a shorter time and reduces solvent consumption as compared to other extraction techniques such as maceration, percolation and the use of super critical fluids [69]. The extraction using enzymes has also showed an effective isolation of phlorotannins, as the enzymes were found to damage the cell walls and release the compound located inside the algal cells. The enzymatic extraction provides the yield of 21–38% from *Sargassum polycystum*, whereas the conventional solid-liquid extraction provides only 3–15% yield [72]. The enzyme-assisted extraction using enzymes, such as termamyl enzyme, cellulose enzyme, viscozyme enzyme with concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% (v/v); varied time intervals were used to isolate phlorotannin from *Sargassum duplicatum* [13]. These methods together with the advancement in technology over a time period provide efficacy and potency.

Ultrasound, Super Critical Fluid and Microwave-assisted Extraction

Nowadays, large-scale extraction at low costs was made possible with the help of recent technologies such as ultrasound- and microwave-assisted methods. The compound of interest can be extracted by causing physical damage to the cell membrane by cell wall disruption, which is the more efficient way in isolating cell wall bound as well as phlorotannin molecules that are present inside the cells [40]. High molecular weight phlorotannins can be isolated by using these techniques in which the mass transfer facilitates the destruction of algal cell walls [5]. This disruption causes particle size reduction and makes the frequent contact between the compound and the solvent used [74]. Similarly, the configuration of various phlorotannins was detected by means of microwave-assisted extraction. It is considered as an efficient extraction process that, compared to others, delivers greater purity with less time in processing [75].

Eleven different brown seaweeds were quantitatively investigated for phlorotannins with the use of ultrasound-assisted extraction. This method uses the frequency range of 20 to 100 kHz that facilitates faster extraction. This technique provides a high yield of phlorotannins at a low cost, high efficiency and in an eco-friendly manner [90]. The pressurized liquid extraction provides higher productivity of phlorotannin compounds from *Ascophyllum nodosum*, *Pelvetia canaliculata*, *Fucus spiralis*, and *Ulva intestinalis* in comparison to the traditional extraction method [38]. Phlorotannins from *Fucus vesiculosus* were extracted with the use of microwave assistance; a hydro-ethanolic mixture was used as a solvent. With the optimal

conditions of 57%-ethanol (v/v) and a temperature of 75°C for 5 min, phlorotannin constituents were isolated and found to be 9.8 ± 1.8 mg PGE/g DW extract [4]. Similarly, a high quantity of phlorotannins was extracted using microwave-assisted extraction under the condition of 52% ethanol and 163 W microwave output power for 65 minutes; the obtained concentration was 5.59 ± 0.11 mg PhE/g [73].

High molecular weight phenolic compounds were separated from *Ascophyllum nodosum* using ultrasound-assisted extraction process and analyzed by mass spectroscopy [82]. The extraction of phlorotannin from *Silvetia compressa* was enhanced with the help of ultrasound power density [91]. Some phlorotannins were reported for extraction using Supercritical carbon dioxide extraction method, which uses eco-friendly carbon-dioxide [79]. This type of extraction was done in marine brown algae such as *Sargassum vulgare*, *Sargassum muticum*, *Porphyra/Pyropia spp.*, *Undaria pinnatifida*, and *Halopithys incurva*. Recently, this procedure has been recognized as more acceptable, especially for the extraction of phenolic compounds at its supercritical state. Supercritical carbon-dioxide solvent extraction of phenolic compounds was used individually or in combination with organic solvents, which delivers a higher yield [61, 23]. These advanced methods of extraction not only give a higher yield but are also suitable for thermolabile molecules like phlorotannins and also reduce the extraction time and the solvent volume [5, 41, 72].

Extraction based on Eutectic Solvents

The emerging method in the extraction of phenolic compounds from seaweeds was applied using natural deep eutectic solvent (NADES) in which the combined solvents having lower melting point was used. This method offers environment sustainability and efficient extraction similar to other modern techniques [27]. The predominantly used NADES for polyphenol extraction was based on choline chloride along with the organic solvents such as citric acid, lactic acid, and acetic acid. The mechanism involved is the hydrogen donating ability of polyphenols that interact with the hydrogen acceptors like choline chloride. The hydrogen donors in the case of phlorotannin compounds is the hydroxyl group present in the structure.

The suitable solvents could be approximately predicted via an *insilico* technique called quantum conductor-like screening model for real solvent (COSMO-RS) and can be further confirmed by experimental analysis [71]. For example, the total polyphenols extracted from *Fucus vesiculosus* and *Ascophyllum nodosum* with the help of choline chloride and lactic acid were obtained in the ratio of 1 : 1, 1 : 2, or 1 : 3 [66]. Similarly, the extraction of phlorotannin from *Fucus vesiculosus* with NADES and water showed efficient extraction and potential antioxidant capacity

[67]. Nicholas et al. reported the DES extraction of phloroglucinol dehydrate using choline chloride and water. The solubility of phloroglucinol from brown seaweeds showed proficient extraction, which was highly beneficial by using DES-extraction technique [31].

PURIFICATION, QUANTIFICATION, AND CHARACTERIZATION

After the extraction step, quantification and characterization are required in order to isolate the purified phenolic compound. Depending on the property and nature of the compound, various types of procedures could be carried out. The quantification and assessment of the phenolic content from brown seaweeds was traditional and mostly performed by colorimetric assays such as Folin–Ciocalteu (F-C), Folin–Denis, or Prussian blue assays [58]. Reports on quantification of some marine brown algae such as *Fucus spiralis*, *Sargassum fusiforme*, *Macrocystis pyrifera*, and *Laminaria digitata* with the use of the F-C assay were published by Ford et al. [30], who also mentioned different analytical methods for extraction and characterization of phlorotannins.

Spectroscopic and Chromatographic Techniques

In the FTIR (Fourier Transfer Infrared) spectrum, the intensity of the absorption frequencies (cm^{-1}), representing particular function groups (C=C, O–H, C–H) reveals the presence of phenolic compounds. The marine brown algae *Durvillaea antarctica* and *Hormosira banksii* were analyzed for the presence of phenolic compounds using FTIR [24]. The FTIR spectrum of kelp phenolic compounds was determined to evaluate the presence of phlorotannins [8]. The low-molecular-weight phlorotannins were characterized by the method of NMR (Nuclear Magnetic Resonance) spectroscopy along with the fast atom bombardment ionization – mass spectrometry (FAB-MS) and electrospray (ESI-MS) ionization method. In comparison of the NMR spectrum with other spectroscopy methods, NMR has a lower mass sensitivity [11]. The technique named High-Resolution Magic Angle Spinning (HRMAS) was exclusively used for analysis of phlorotannins from brown seaweeds, for example from *Cystoseira tamariscifolia*.

The chromatographic techniques, such as column chromatography, high-pressure liquid chromatography (HPLC), and thin-layer chromatography (TLC) are used for separation, isolation, identification, and purification of phenolic compounds, in particular phlorotannin [58]. HPLC, an automated procedure along with the proper detection system, is widely used for separation, purification, and characterization of many chemical compounds [94]. This method requires minimal time duration and is a very rapid method even with the small quantity of the sample [78]. The most frequently used technique for the char-

acterization of phlorotannins was HPLC-HRMS (high resolution mass spectrometry) as this method gives fast and accurate results [59]. There are many reports based on the HPLC procedure for several marine brown algae. Separation of nine phenolic compounds such as gallic acid, 4-hydroxybenzoic acid, catechin hydrate, epicatechin, catechin gallate, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and pyrocatechol from edible marine brown algae *Eisenia arborea*, *Sargassum fusiforme*, *Laminaria japonica*, and *Undaria pinnatifida* were studied by Machu et al. [55]. Chromatography–mass spectrometry procedure has gained a special interest in the study of some complex phenolic compounds. Identification and quantification of some phlorotannins were accomplished via reverse-phase liquid chromatography (RP-HPLC).

LC-MS, GC-MS and UHPLC-ESI-MS

The characterization and quantification of phenolic compounds were exhibited via the pairing of liquid or gas chromatography with mass spectrometry [33, 41]. Using liquid chromatography–mass spectrometry (LC-MS) technique and LC-DAD-ESI-MS/MS, the phlorotannins can be isolated according to the degrees of polymerization from some marine brown algae, such as *Durvillaea antarctica*, *Lessonia spicata*, and *Macrocystis pyrifera* [68]. Wherein, gas chromatography–mass spectrometry (GC-MS) determined the presence of coumarin and flavones from the crude extracts of *Padina tetrastratica* [56]. The identification and analysis of phlorotannin from marine brown algae based on LC-MS/MS and UHPLC were described by many authors [38, 52, 62, 78, 85].

Phlorotannins with a high degree of polymerization were determined using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) and high resolution mass spectrometry (HRMS). Lately, ultra-performance liquid chromatography mass spectrometry (UPLCMS) technique was used to display the polyphenolic profile of some marine brown algal species [94]. The presence of phloroglucinol from the marine brown algae *Sargassum wightii* has been detected by using MALDI-TOF-MS [45]. Combination of ultrahighperformance liquid chromatography–electro spray ionization tandem mass spectrometry (UHPLC-ESI-MS) with MALDI-TOF-MS can be used for characterization of phenolic compounds according to the size and isomerism [78]. This procedure was applied in order to recognize and characterize about 22 phlorotannins extracted from *Fucus* species [54]. The complication in isomerism and rapid profiling of the phlorotannins has also been studied with the help of Ultra-High-Performance-Liquid-Chromatography (UHPLC) [36]. The components of the phlorotannin extract from *Silvetia compressa* were analyzed using HPLC-MS-TOF, and different phe-

nolic compounds such as fuhinol, eckol and phloroglucinol derivatives were identified [91]. The purification and characterization of phlorotannins from *Sargassum* species with various molecular weights were performed using a modified UHPLC-QQQ-MS method [52]. This method was also used for determination of the molecular weight of 42 different phlorotannins from marine brown algae, especially from *Sargassum fusiforme* [52]. Solid-phase extracts of *Ascophyllum nodosum* and *Saccharina latissima* were purified using UHPLC-HRMS; this provides rapid profiling of phlorotannins according to the degree of polymerization. HILIC (Hydrophilic Interaction Chromatography) technique was found to be an effective method for separation of low-molecular weight phlorotannins [85]. Four different phlorotannin derivatives were identified [80]. The method of liquid-liquid fractionation of *Fucus spiralis* with the use of organic solvents yields phlorotannin-rich extracts [3]. These techniques also help for assisting the separation of complex phlorotannins, which are covalently bound to polysaccharides [90]. The techniques mentioned above are used for separation, characterization and purification of phlorotannins from marine brown algae in order to explore their various biological effects.

ANTIVIRAL POTENTIAL OF PHLOROTANNIN

The onset of the pandemic situation, evolving the nature of new strains of harmful viruses like COVID (Corona virus) variants, has created a great impulse for discovering suitable target molecules against viruses. As the viruses tend to recur into various states, the discovery of novel operative drug molecules is becoming persistent. The in vitro antiviral activities are assessed via various antiviral assays. The antiviral assays were performed in order to investigate potential antiviral compounds in preventing several viral infections. The phlorotannin compounds were reported for exhibiting strong antioxidant properties, which in turn aid in suppressing the growth of viruses. This property will modify the oxidative environment, which creates an unfavorable condition for the virus to multiply. Oxidative stress may also contribute to the reproduction of viruses in diseased cells [77].

The antiviral property of phlorotannin has lately gained focus and are mostly studied polyphenolic compound in vitro against harmful viruses [92, 96]. The three stages of the development of a viral infection are its entry into the host cell, attachment to the surface proteins and replication of the genetic material, thereby multiplying itself into several copies. The phlorotannins from brown seaweeds were found to affect the viral cells in all the three stages of the cell cycle [1]. The existing and synthetic drug molecules display narrowed action with adverse reactions to some extent. The natural compounds with negligible

side effects have a broad action towards inhibition of virus growth [42].

Phlorotannin Action Towards COVID

Phlorotannin derivatives were found to be dominant in inhibiting viral proteins. PLpro protein is one of the prominent proteins responsible for processing viral proteins for multiplication. PLpro proteins present in acute respiratory syndrome corona virus (SARS-CoV) was found to be inhibited by the phlorotannin compound isolated from *Ecklonia cava* [72]. Ethanol extraction was done to obtain phlorotannin derivatives such as dieckol, eckol, phlorofucofuroeckol A dioxinodehydroeckol, 2-phloreckol, 7-phloreckol, fucodiphlorethol, and triphlorethol A. The antiviral potential of different phlorotannins was evaluated for every compound, which displayed distinctive ability. Among them, dieckol was reported to have the minimum activity ($IC_{50} 2.7 \pm 0.6 \mu M$) and triphlorethol A ($IC_{50} 164.7 \pm 10.8 \mu M$) showed the maximum activity towards eliminating viruses. This information indicates that phlorotannin could be established as a promising antiviral compound.

The porcine epidemic diarrhea (PEDL) is one the members of coronavirus family, which were reported for causing death in newborn piglets. This virus was targeted and inhibited by an ethanol extract of *Ecklonia cava*, which consists of phloroglucinol, eckol, 7-phloreckol, phlorofucofuroeckol and dieckol, respectively. The antiviral effect of five different phlorotannins has a prominent effect in inhibiting the PEDL viruses. The activity of phloroglucinol seems to be quiet, whereas other phlorotannins showed activity, as dieckol ($16.6 \pm 3.0 \mu M$), 7-phlorofucofuroeckol ($18.6 \pm 2.3 \mu M$), and eckol ($22.5 \pm 2.3 \mu M$), respectively. According to the obtained results, the polymerization and structural arrangements play an important role in the antiviral activity [49].

Phlorotannin Action Towards other Viruses

Human papilloma virus (HPV) is capable of causing genital infections and are related to cervical cancer. The inhibitory effect against this harmful HPV was studied using the ethyl acetate fraction of phlorotannin compounds from *Eisenia bicylis*. The chromatographic fraction showed the presence of eckol, 8,8'-bieckol, 6,6'-bieckol, and phlorofucofuroeckol A in the extracted fraction. The antiviral activity was analyzed using 293T cell lines. The bioluminescence assay was performed and showed the inhibitory potential of $50 \mu g/mL$ (IC_{50}). The phlorotannin compounds were found to be effective against both HPV 16PVs (type16-pseudovirions) and HPV 18PVs and can be a prominent anti-HPV molecule [46].

The murine norovirus (MNV) is associated with an enterovirus type virus, which causes severe symptoms and is spread via contaminated water and food.

According to the reported data, the ethyl acetate fraction of *Eisenia bicylis* gives phlorotannin molecules, dieckol and phlorofucofuroeckol-A. These compounds were examined for exhibiting antiviral properties. From the experimental results, phlorofucofuroeckol-A exhibited a strong anti-MNV effect, as compared to dieckol with the EC50 value of 0.9 μM [26, 50]. This study suggested that phlorofucofuroeckol could be a promising antiviral compound. It is also mentioned that the phlorotannin compounds inhibit the entry of virus into the host cells. Ziad *et al.* reported that the aqueous extracts of two marine seaweeds, *Cystoseira myrica* and *Ulva lactuca* showed a negotiable antiviral property. The phenolic compounds obtained from the extract were tested against few virus types such as hepatitis A virus (HAV-H10), Coxsackie B4 virus, herpes simplex virus types-1 (HSV-1) and type2 (HSV-2). The results based on MTT assay for cytotoxicity and neutralization assays showed that the phenolic compounds exhibited antiviral and cytopathic effect performed on Vero cell lines [98].

The phenolic compound 8,4'-dieckoll, which is a derivative of phlorotannin possesses a wide range of biological activities. It was extracted from the marine seaweed *Ecklonia cava* and has been evaluated for its anti-viral property against human immunodeficiency virus type-1 (HIV-1). The results indicated that 8,4'-dieckol could inhibit the activity of HIV-1 reverse transcriptase (RT) enzyme with the inhibition ratio of 91% at the concentration of 50 μM . The entry of HIV-1 was also found to be suppressed and was reported as a potential anti-viral compound [28]. The anti-viral activity of phenolic extracts from different marine brown algae, such as *Ecklonia arborea* and *Solieria filiformis*, were evaluated for their activity against Measles virus (MeV). The MTT assay for cytotoxicity and syncytia reduction assay for antiviral activity were performed. Extracts from the both algae showed a high level of efficiency and a low cytotoxicity, as compared to that of the ribavirin, standard. The antiviral effects of the phenolic extracts were confirmed by qPCR, they were found to inactivate the viral particle [63]. The enzymatic extracts of *Sargassum muticum* were evaluated for anti-viral potential against Herpes simplex virus type 1 (HSV-1) and cytotoxicity activity using African green monkey kidney cells (Vero cells) [72]. According to the review article, phlorotannin derivatives exhibited a promising antiviral potential in *in vitro* analysis. Some other reports regarding antiviral activities are given in Table 2.

Immunomodulating Activity for Inhibiting Viral Growth

As the immune system plays a major role in eliminating viruses, the anti-inflammatory property of immunomodulators plays a vital role in modifying the immune system by provoking innate and adaptive immune responses thereby inhibiting viral growth. Immunomodulation is established by the enhance-

ment of an immune regulatory mechanism as well as inhibition of uncontrolled immune responses by fighting viral infections. A type of phlorotannin, diphlorothohydroxycarmalol (DPHC) from *Ishige okamuarae* inhibited the nuclear translocation of NF- κ B pathway and down regulated the formation of IL-6 [44]. The low molecular weight phlorotannins extracted from *Fucus vesiculosus* exhibited an effective immune modulating activity by suppressing the nitric oxide (NO) production in LPS-induced macrophages. The sample at the concentration of 100 $\mu\text{g}/\text{mL}$ reduced the NO production by 85%. According to the results, phlorotannin could be a good anti-inflammatory molecule from natural source [16].

Dong *et al.* reported that phlorofucofuroeckol A, a dieckol from brown seaweeds was found to have anti-inflammatory activity better than that of Epigallocatechin gallate (EGCG). The extract at all concentrations inhibited NO production and exhibited no cytotoxicity to the cells; this effect was assessed by carrying out immunomodulatory activity assay, which confirmed an effective therapeutic agent even at low concentrations [25]. *Ecklonia cava* and *Sargassum horneri* were evaluated for anti-inflammatory activity on LPS-stimulated RAW 264.7 cell lines. The extraction of 70% ethanol from both seaweeds were evaluated for their immune responses on cytokine production, protein and gene expression. The activity was enhanced when both extracts were combined together, compared to the results for results for individual extracts. The result showed a 8:2 combination of *Ecklonia cava* and *Sargassum horneri* extracts that effectively inhibited the pro-inflammatory responses [7].

Eckol from *Ecklonia* exhibited an immune modulating potential, in tumor bearing mice it was found to activate phagocytosis, CD11c⁺-dendritic cells (DC), CD4⁺/CD8⁺ T lymphocyte ratio, tumor-specific Th1, and cytotoxic T lymphocytes *in vivo* [60]. The enhancement of phagocytosis in human blood leukocytes was achieved via polyphenols from *Fucus vesiculosus*. The polyphenols were tested *in vivo* in outbred white mice by injecting intra-peritoneally, at the concentration of 5pg/ml with 1 mL saline, which were found to establish immune modulation [12]. In addition, dieckol from *Ecklonia cava* exhibited in PC12 cells effective anti-inflammatory activity by reducing the activity of p38, Extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinases (JNK). Hence, phlorotannin can be a potential immune modulating compound and a therapeutic agent [51]. These phlorotannin derivatives from marine seaweed possess anti-inflammatory and immune modulating activity thereby inhibiting viruses.

Antioxidant Effect of Phlorotannin Towards Viral Growth

Reactive oxygen species are capable of damaging cellular functions such as DNA and protein damage,

deactivation of enzymes, gene alteration and lipid peroxidation, which in turn also creates a favourable environment for the replication of viruses. The phlorotannin molecules, along with their antiviral property, were also found to possess an antioxidant property. To scavenge the free radicals responsible for causing oxidative damage, a potential antioxidant effect is necessary to maintain healthy body cells. There are several reports on powerful antioxidant potential of phlorotannins obtained from marine brown algae. Oxidative stress leads to the formation of free radicals and reactive oxygen species, which causes progression of many harmful viral infections. The property of antioxidants to modify the oxidative environment not only eliminates viruses but also ensures the molecules to be toxic-free as a pharmaceutical preparation [29].

For example, phlorotannins from *Carpophyllum flexuosum*, *Carpophyllum plumosum* and *Ecklonia radiata* showed a higher DPPH antioxidant capacity of 62.1 mg gallic acid equivalents (GAE)/g dry weight of seaweed; and phlorotannin from *Fucus serratus* showed a strong antioxidant ability of 63.9 ± 0.74 mg trolox equivalent/g performing FRAP assay [75]. Phlorotannin isolated from *Sargassum duplicatum* was studied for antioxidant activities after purification. The enzyme-assisted extraction of phlorotannin antioxidant potential of 11.17 ± 0.28 mg ascorbic acid equivalent/g dry weight, reducing power activity of 11.09 ± 0.24 mg FeSO₄ equivalent/g dry weight. Lipoxigenase enzyme-inhibition activity was performed and showed the antioxidant activity as 66.19 to 87.09 μ M linoleic acid equivalent/100 μ L of the sample, respectively [13].

The crude extract and dichloromethane (DCM) fraction from the marine brown alga *Cystoseira trinodis* was evaluated for antioxidant capacity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity. The DCM fraction was the solvent-extracted sample using methanol, which showed antioxidant activity of 69.62% [81]. The solvent extracted fraction of phlorotannin from five different seaweeds, such as *Saccharina latissima*, *Alaria esculenta*, *Laminaria digitata*, *Fucus vesiculosus*, and *Ascophyllum nodosum* showed a remarkable antioxidant potential in comparison to standard antioxidants, butylated hydroxytoluene and ascorbic acid [53]. DPPH radical scavenging assay was performed, in which ethanol fraction of *Agarum turneri* exhibited the highest antioxidant capacity evaluated as 38.8 mg ascorbic acid/g and 2506.8 μ M Trolox equiv/g dry algae [5]. Besednova [9] analyzed phlorotannin from marine seaweeds as an antimicrobial agent and found it to be a promising candidate against gram-negative and gram-positive bacteria. Several algal species are have been explored.

STORAGE PROPERTIES OF PHLOROTANNIN

Phlorotannin, like polyphenols, was revealed to be sensitive to longtime storage after extraction. The factors affecting storage include dissolved oxygen, light, temperature and time [64]. According to Coung et al. [21], the storage of phlorotannin from different *Sargassum* species in polythene bags at 30°C for two years significantly reduces the content of phlorotannin. In addition, the antioxidant property of *Sargassum dentocarpum* almost disappeared after the storage period of 18 months; the experiments were given in detail by Cassani et al. [14]. An innovative method called encapsulation was found to be a better alternative to store these sensitive phlorotannin compounds in terms of micro or nano encapsulation. This method of storage as coating, shells and wall material will protect the active compound from degradation [64]. Surendhiran et al. validated a technique of nanofibre encapsulation for the storage of phlorotannins, which were found to be stable [87]. Another report describes storage of phlorotannins from the brown seaweed *Fucus vesiculosus* with the use of electrosprayed capsules using dextran, which exhibited good storage property [37]. This method protects phlorotannin compound from light, temperature and oxygen. These studies suggest that the proper storage of phlorotannins will make them active and stable.

CONCLUSION

According to the data and reports published, phlorotannin from the marine natural source possesses various functional antiviral molecules with various biological activities that could be a promising therapeutic approach and nutraceutical molecule and a disease preventive agent. As the rate of infections are increasing exponentially, the discovery and development of new effective drugs become essential. In vitro results revealed that phlorotannin could be effective against a wide range of infections. Moreover, intense research is required to gain knowledge about the structural complexity, extraction efficiency and the methods of purification. Isolation of phlorotannin can be performed using both classical and modern techniques. The results obtained in vitro are not sufficient for dealing with the immune response of the human body, whereas in vivo studies are mandatory to overcome the limitations. They also help in gaining information about the bioavailability, efficacy and safety of phlorotannin and make them a novel therapeutic drug for preventing infections and disorders. The marine environment can provide a promising lead for the development of a new generation of drugs. Thus, phlorotannin from brown seaweeds could not only be used for treatment but also for prevention of particular disease conditions.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. This article does not describe any research using humans and animals as objects.

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