Obando JMC, et al., J Aquac Fisheries 2024, 8: 087
DOI: 10.24966/AAF-5523/100087

HSOA Journal of Aquaculture & Fisheries

Research Article

Diterpene Biosynthesis of Seaweed *Canistrocarpus cervicornis*: A Potential Method by Diterpene Obtention in Brown Algae Using Aqueous Extract as an Enzyme Source

Johana Marcela Concha Obando[1,2]*, Thalisia Cunha dos Santos[2], Marcelo Bernardes[3], Nívia Nascimento[3], Roberto Campos Villaça[1], Elisabete Barbarino[1], Valeria Laneuville Teixeira[2,4] and Diana Negrão Cavalcanti[1,2]

[1]*Universidade Federal Fluminense, Programa de Pós-Graduação em Biologia Marinha, Instituto de Biologia, Niterói, RJ, Brazil*

[2]*Laboratório de Produtos Naturais de Algas Marinhas (ALGAMAR); Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brazil*

[3]*Universidade Federal Fluminense, Programa de Geoquímica, Central Analítica de Facilidades Geoquímica, Instituto de Química, RJ, Brazil*

[4]*Universidade Federal Fluminense, Programa de Pós-Graduação em Ciências e Biotecnologia, Instituto de Biologia, Niterói, RJ, Brazil*

Abstract

The Dictyotaceae family's chemistry involves producing several diterpenoids with different skeletons. These molecules have aroused great interest in the pharmaceutical and biotechnology field from a drug discovery point of view. Evidence for the use of precursors, cyclization processes, and enzymes involved in diterpene production in seaweed o has poorly been explored, and few studies, including biogenetic proposals, have been reported. The main objective of this work is to highlight the methodological advances obtained in this line of research after several experiments' optimization, in addition, our presenting the first satisfactory proof after monitoring using Gas Chromatography-Mass Spectrometry (GC-MS) the enzymatic cyclization processes on a small scale and in reaction times of less than 2 hours. The 2 h reaction time showed a precursor (GGPP) consumption trend of 56.21%, simultaneously, the relative abundances of the main diterpenes 4,14-dihydroxydolastane-1(15),7,9-triene (1), 4-acetoxy-14-hydroxydolastane-1(15),7,9-triene (2), 4,7-diacetoxy-14-hydroxydolastane-1(15),8-diene (3) and 4-acetoxy-9,14-dihydroxy-1,9-dolastane-1,9-diene (4) were increasing. Finally, new signals in the chromatogram with retention times 32.391 (M+ 281) and 32.669 (M+ 281) with spectra similar mass to diterpenes were considered evidence of enzymatic activity. Our results demonstrate the biosynthetic processes and cyclization of the precursor extracted from annatto seeds to obtain group II diterpenes from the seaweed *Canistrocarpus cervicornis* using the chemical-enzymatic arsenal of its aqueous extract

Keywords: Biosynthetic pathway; *Canistrocarpus*; Diterpenes; Enzymatic activity; Natural products

***Corresponding author:** Johana Marcela Concha Obando, Universidade Federal Fluminense, Programa de Pós-Graduação em Biologia Marinha, Laboratório de Produtos Naturais de Algas Marinhas (ALGAMAR); Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil, Email: mc1226@gmail.com

Citation: Obando JMC, Santos TCD, Bernardes M, Nascimento N, Villaç RC, et al. (2024) Diterpene Biosynthesis of Seaweed *Canistrocarpus cervicornis*: A Potential Method by Diterpene Obtention in Brown Algae Using Aqueous Extract as an Enzyme Source. J Aquac Fisheries 8: 087.

Received: June 03, 2024; **Accepted:** June 13, 2024; **Published:** June 20, 2024

Introduction

Marine organisms are a rich source of structurally novel and biologically active metabolites, and algae have been widely documented as a source of both primary and secondary metabolites. Brown algae of the family Dictyoteceae, produce diterpenes with diverse skeletons that exhibit a wide range of biological activities; many of them have pharmacological and biological activities and are therefore of interest to medicine and biotechnology [1-3]. Chemical investigation of the genus *Canistrocarpus* has led to the isolation of a variety of diterpenes such as dolastane, secodolastane, and oxodolastane with different biological/ecological activities [4,5]. Among its activities studied are antiviral [6-8], antibacterial [9], antioxidant [10], antifoulant [11], anti-inflammatory [12]. In such a manner, there is currently an urgent need for options with a sustainable perspective to exploit these algae as a source of bioactive compounds, thus, studies evaluating the profile of diterpenes *in vitro* cultivation have been advanced to explore these molecules [13,14]. Nevertheless, it is essential to invest in other approaches and methodologies for the recovery of these compounds on a commercial scale. Hence, the biosynthetic process assays are a way novel and promissory to the production of diterpenes.

All terpenes are synthesized from the two universal building blocks Isopentenyl Pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can be formed from mevalonate (MVA) or 2-C-methyl-d-erythritol-4-phosphate (MEP). Prenyltransferase (PT) and terpene synthase (TPS) are key enzymes in the formation of the basic carbon skeletons of the terpenes [15]. Diterpenes are a class of C20 compounds formed from the isoprenoid precursor geranylgeranyl diphosphate (GGPP), which is itself synthesized from DMAPP and three IPP molecules by catalysis of geranylgeranyl diphosphate synthase. Diterpene synthases (DTSs) are the enzymes that frequently catalyze the formation of various products, due to a mechanism involving highly reactive carbonization intermediates [16]. DTSs act in two different ways: by ionization of diphosphate catalyzed by class I enzymes, and by protonation of the substrate at the 14,15 double bond of geranylgeranyl diphosphate; the reaction is catalyzed by class II enzymes [17].

Despite the tremendous progress in other biological groups such as plants and bacteria in the study of terpenoid biosynthesis, there is little work that highlights and examines the metabolic pathways involved in the production of terpenes in algae, and, there is little experimental evidence to support this, for example, the green algae do not possess the genes for the MVA pathway and their sesquiterpene and triterpene building blocks are consequently derived from the MEP pathway. However, red algae such as *Laurencia* species retained both IPP pathways during their evolution and used both biosynthetic routes [18]. Recent studies have reported the identification of several terpene synthase genes from the transcriptomes of the red macroalgae *Laurencia pacifica* and *Laurencia dendroidea* in the class Florideophyceae [19]. This opens an interesting field of research for the detection of such metabolic pathways in diterpenes in seaweed. Specifically for algae of the *Dictyotacea* family, no experimental evidence for the formation of these molecules, although has been described, as a biogenetic pathway for diterpene formation, whereby the precursor geranylgeraniol phosphate (GGPP) can undergo the first type of cyclization between carbons 1 and 10 (group I diterpenes skeleton), between carbons 1 and 11 (group II diterpenes skeleton), and between carbons 2 and 10 (group III diterpenes skeleton) of the germacrane-type precursor belonging to group I [20].

Notwithstanding the diversity of terpenes in brown algae, little is known about how they are biosynthesized, and there are no experimental approaches, only pioneering work on terpene biosynthesis in plants [21,22]. On the other hand, despite the fundamental role of geranylgeraniol diphosphate (GGPP) as an essential metabolic hub for plant growth, development, and acclimation, there are few studies on its role as an enzyme precursor in terrestrial plants and even rarely in algae [23,24]. Therefore, this study aimed to investigate the *in vitro* reactivity of the precursor geranylgeraniol (GGOH) and its phosphate equivalent at different reaction times in *C. cervicornis* and to understand the biosynthetic potential of the aqueous extract containing active cyclase enzymes from *C. cervicornis.* We established a promising method for obtaining an enzymatic extract of brown seaweed capable of using an enzymatic precursor obtained from natural and low-cost sources. The evidence shown here will be important to continue the biotechnological potential of these important molecules.

Material and Methods

Biomass treatment

Specimens of *C. cervicornis* were collected on Ilha Grande (23°09'18.6" S 44°20'37.5" W) in Rio de Janeiro State, Brazil. Collections were made by snorkeling in shallow water at depths of 2.0 to 5.0 m. Algae were separated from sediments, epiphytes, and other associated organisms and washed with seawater. The collected biomass was immediately placed in a cryogenic cylinder (liquid nitrogen - N_2) to preserve active physiological function. In the laboratory, the samples were divided into two fractions: a dry fraction at room temperature (approximately 28-30 °C for 7 days) for organic extraction and chemical confirmation, and another fraction kept in an aeration system for aqueous extraction. Voucher specimens were deposited in the herbarium of the Universidade Federal do Estado do Rio de Janeiro - (Herbário Prof. Jorge Pedro Pereira Carauta) under the number HUNI 6810.

Crude extracts

An aliquot of 2 g of dry alga was exhaustively extracted with CH_2Cl_2 at room temperature for 120 h, and the solvent under reduced pressure yielded a brownish residue: 82 mg (4.4%) and 20 mg (1.1%). A small aliquot (5 mg) from each fraction was dissolved in $CDCl_3$ to obtain the ^1H NMR (500 MHz) spectrum in a Varian-Unity Plus 500 using trimethylsilyl as the internal reference and compared to data previously reported [4].

Obtaining and identifying the precursor GGOH

The methodology was adopted by Costa and Chaves (2005) [25]. Accordingly, dry seeds (87.39 g) of *Bixa orellana* were completely extracted in n-hexane (100 ml) for one month (720 h) at room temperature. Evaporation of the solvent was under reduced pressure (Buechi, model R 114) giving an orange residue (4% of the dry weight of the seeds). The crude extract (1.0 g) was fractionated on a silica gel 60 for column chromatography (70-230 mesh ASTM) and then mixed with 100% n-hexane to give 5:5 [vol/vol] EtOAc: n-hexane in increments of 10% and eluted with 200 mL, yielding 69 fractions. Fractionation was monitored by thin-layer chromatography (TLC silica gel 60 F254) according to chromatography profiles and analyzed by nuclear magnetic resonance ^1H NMR. The bioactive fraction (F23-25; 165.0 mg) was successively rechromatographed on a silica gel 60 column (230-400 mesh-ASTM) with n-hexane: EtOAc (8:2 [vol/vol]). The column fractions were analyzed and fractions with similar stripe patterns on the TLC (silica gel 60 F254) were pooled to give compound 1 (130 mg). The structure of geranylgeranyl was then determined by ^1H and ^{13}C NMR using a Varian unity plus 500 at 500.00 M in deuteriochloroform ($CDCl_3$). The spectra obtained were analyzed using the MestReNova (version 6.0.2) software and compared with the literature (Jondiko and Pattenden, 1989). The substrate GGPP was obtained by the reaction of GGOH (purified) with a phosphate source [26]. The phosphate source was prepared using 0.5 ml phosphoric acid and 10 ml NaH_2PO_4 solution. H_2O. Aliquots were then taken and Na_2CO_3 was added until the desired pH was obtained.

Enzymatic extraction

The protocol adapted from Lewinsohn et al., (1991) [22] was used. Thus, frozen pieces of algae (3 g fresh weight) were weighed and placed in a small envelope of manila paper and, while still frozen, were macerated. The resulting fine powder was thawed by thorough mixing (vortex mixer) in a chilled extraction buffer solution (10 mL buffer/g fresh weight). The extraction buffer contained 50 mM Hepes (pH 7.8), 5 mM sodium ascorbate, 5 mM $Na_2S_2O_5$, 5 mM $MnCl_2$, 20 mM $MgCl_2$, and 10% (v/v) glycerol. The mixture was mechanically stirred at 4ºC for 4 hours (Fisatom Mod.710). For the standard extraction procedure, this buffer also contained PVP (1% w/v, Mr = 40,000). Aliquots (usually 2 ml) of the suspension were centrifuged in the Eppendorf microcentrifuge (27,000 g) for 20 minutes at 4°C, and the supernatant was filtered and collected as an enzyme extract. The protein concentration of the extract was determined by the Lowry method [27]. The organic part of the aqueous extract was obtained by extracted with chloroform CH_2Cl_2, evaporated, analyzed by gas chromatography–mass spectrometry (GC-MS), and used as a control 2 (blank) for the analysis of the biosynthetic assays.

Cyclase assay

The protocol adapted from Lewinsohn et al., (1991) [22] was used. Thus, a 1 mL aliquot of the enzyme extract was transferred to a 1.5 mL Eppendorf tube, to which 1 mg of the precursor (12 µM GGPP) was added, the contents were mixed (vortex mixer), and incubated for up to 2 h at 30°C. Cyclase assays were tested at the following

reaction times: 5', 10', 30', 45', 60', 90' (1h 30) and 120' (2 h). After incubation, the contents of the tube were vigorously mixed and briefly centrifuged to separate the phases, and the sample was immediately extracted three times with CH_2Cl_2. Using a glass pipette, the polar portion was carefully removed, and the dichloromethane fractions were transferred to 1.5-mL vials, dried, and later stored with an air-tight Teflon lid and kept in the same properly labeled vials, wrapped with aluminum foil and stored at -18°C ± 2°C until the time of GC-MS analyses. The non-parametric U Mann-Whitney test was used to compare yields of extracts of E1. The Kruskall-Wallis test was used to compare the abundances of GGPH and diterpenes. Test $p < 0.05$. Graphs and tests were generated using GraphPad Prism 8.0.

Diterpene profile

Quali-quantitative identification of biosynthesis and diterpene profile by GC-MS was performed following the protocol described previously by Obando et al 2023 [14]. Briefly, the analysis was performed using an Agilent 7890A/5973C equipped with a model column HP5-MS (30 m long, 0.25 mm in diameter, and 0.25 µm film thickness), and a mass selective detector in electron impact mode (70 eV). The chromatograms and mass spectra obtained in the experiments were analyzed with the help of Openchrom open-source software. The GC-MS spectra obtained from the biosynthesis experiments were compared with Blanks (i) Control 1: the profile of the precursor with the reaction mixture (ii) Control 2: the profile of the enzymatic extract with the reaction mixture. The changes in the spectra of the cyclase assays related to the occurrence of new retention times were examined along with the corresponding mass spectra. In addition, the data on the abundance of diterpenes produced relative to the consumption of the precursor were analyzed. Previously described diterpenes were monitored in the assays [4,28,29].

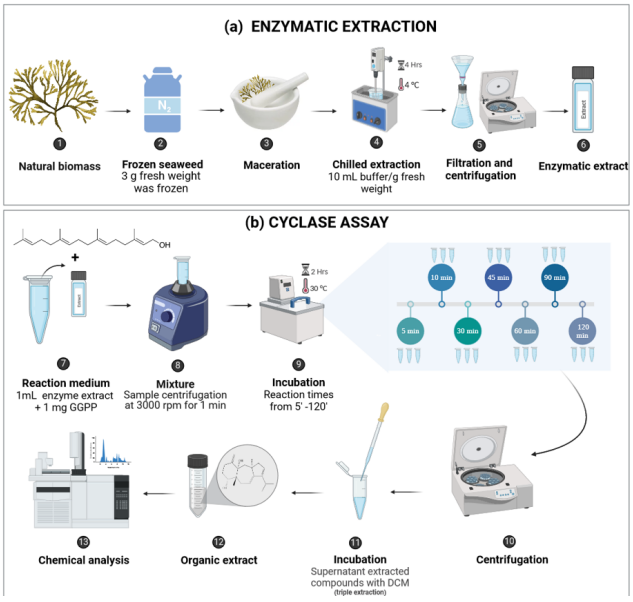

Figure 1: Biosynthesis experiments for brown algae of the genus *Canistrocarpus*. (a) Enzymatic extraction; The steps include (1) obtaining the algae biomass, (2) transporting the biomass in N from the natural habitat to the laboratory, (3) Macerating the biomass using Buffer containing 50 mM Hepes (pH 7.8), 5 mM sodium ascorbate, 5 mM $Na_2S_2O_5$, 5 mM $MnCl_2$, 20 mM $MgCl_2$, and 10% (v/v) glycerol, (4) mechanically stirred and (5) filtration and centrifuged; (b) Cyclase assay: (7-8) reaction mixture (GGP-P+EE), (9) incubation at different times, (10) centrifugation, (11) extracted with dichloromethane (DCM), (12-13) chemical analysis by GC-MS.

Results

Upon examining diverse protocols and conducting multiple experiments, We established a reliable enzymatic extraction method for seaweed of the Dictyotaceae family, using an extraction buffer described in Lewinsohn et al., (1991) [22]. This enzymatic extract reacted with the enzymatic precursor GGPP at two hours allowing us to analyze evidence that indicates the possible production of diterpenes. Thus, we present the graphical scheme of the protocols and steps used in our biosynthesis experiments (Figure 1).

From the organic extract of *C. cervicornis*, ^{1}H NMR analysis revealed the presence of dolastane and secodolastane diterpenes, including the major 4,7-diacetoxy-14-hydroxydolastane-1(15),8-diene and 4-acetoxy-9,14-dihydroxy-1,9-dolastane-1,9-diene. Other studies obtained similar results with signs of dolastanes and secodolastanes. This comparison confirms the production of group IIB diterpenes, according to the biogenetic proposal [4,30]. In the GC-MS spectra analysis of the aqueous extract of seaweed were identified the follow diterpenes: 4,14-didroxy-dolastane-1(15),7,9-triene (1); 4-acetoxy-14-hydroxy-dolastane-1(15),7,9-triene (2); 4,7 diacetoxy-14-hydroxydolastane-1(15),8-diene (3); 4-acetoxy-9,14-dihydroxy-1,9-dolastane-1,9-diene (4); Acetoxy Isolinearol (5). The spectroscopic data for GGOH, including the monitored diterpenes, are detailed in Tables 1 and 2 for the aqueous extract. It is noteworthy that the protein concentration in both samples was 0.011 mg/ml ± 0.01, as described by the equation y = 0.0113x - 0.002.

In this study, GGPP registered an abundance of 90.34 in Control 1 where the precursor was in contact with the other elements of the reaction mixture and without an enzyme pool (Figure 2A). The presence of the alcohol and/or phosphate versions can be confirmed by other analytical methods, such as determining total radioactivity by liquid scintillation counting [31] or under ideal GC-MS conditions to detect proximal differences between these molecules. Second, Control 2 was the aqueous extract (enzyme pool) exposed to the other

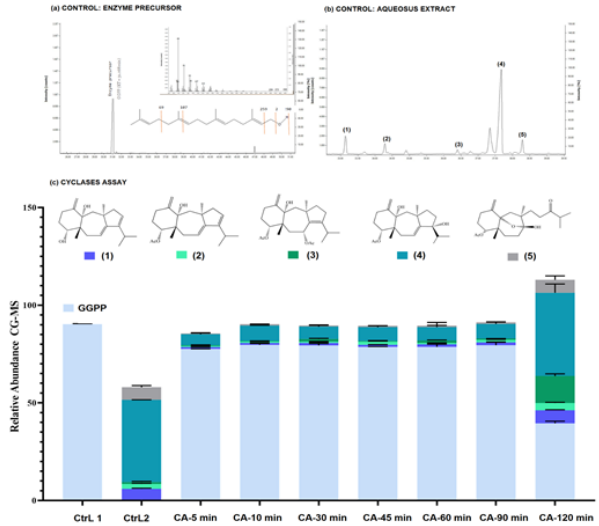

Figure 2: Cyclase assay and controls. (a) Reaction control 1, Enzyme precursor from the seeds of *Bixa orellana*; (b) Reaction control 2, Aqueous extract of the alga *C. cervicornis* used as an enzyme source for cyclase reactions.

elements of the reaction mixture. In this blank, some of the major diterpenes of the alga *C. cervivornis* were detected in small amounts (Figure 2B).

The numbers in the chromatogram represent the major diterpenes identified as chemical constituents of the aquatic extract of *C. cervicornis*: 4,14-dihydroxy-dolastane-1(15),7,9-triene (1); 4-acetoxy-14-hydroxy-dolastane-1 (15),7,9-triene (2); 4,7 diacetoxy-14-hydroxydolastane-1(15), 8-diene (3); 4-acetoxy-9,14-dihydroxy-1,9-dolastane-1,9-diene (4); Acetoxy Isolinearol (5) (Oliveira et al., 2008). C. Relative abundances of control, reaction times of cyclase assay, and precursor enzymatic. After 120 minutes, there is a trend toward a decrease in GGPP and a representative increase in 4,7-diacetoxy-14-hydroxydolastane-1(15),8-diene (3); all bars represent the mean standard deviation (+/- SD, n=3).

Observing the abundance of GGPP and diterpenes in control samples and reaction products over time revealed changes that may indicate the precursor's consumption for diterpene production. The reaction time of 120 min showed a trend of consumption of GGOH of 56.21% and a trend of increase in the relative abundance of diterpenes, 4-acetoxy-14-hydroxy-dolastane-1(15),7,9-triene (2), 4,7-diacetoxy-14-hydroxydolastane-1(15),8-diene (3) and 4-acetoxy-9,14-dihydroxy-1,9-dolastane-1,9-diene (4). It is worth noting that the diterpene (3) increased from an abundance of 0.999 ± 0.30 at blank to 13.78 ± 1.14 at 2 hours. The diterpene (2) recorded in the aqueous extract control at 2.48 ± 0.32 showed a slight increase to 3.75 ± 0.35 at 2 hours, which, together with the decrease in GGPP, may indicate the consumption of the precursor for the production of these diterpenes. Significant differences were recorded only in the compared relative abundances of the enzyme precursor GGPP and the diterpene 4,7-diacetoxy-14-hydroxydolastane-1(15),8-diene (3).

C/H		geranylgeraniol
	δC [a]	δH (nº H; *m*; J Hz) [b]
C1	59.62	4.15 (2H; *d*; 6.9)
C2	123.52	5.42 (1H; *tdd*; 7.0; 2.4; 1.2)
C3	140.06	
C4	26.97	2.05 (2H; *m*)
C5	39.77	2.05 (2H; *m*)
C6	123.98	5.10 (1H; *m*)
C7	135.59	
C8	26.84	2.05 (2H; *m*)
C9	39.89	2.05 (2H; *m*)
C10	124.38	5.10 (1H; *m*)
C11	135.17	
C12	26.53	2.05 (2H; *m*)
C13	39.93	2.05 (2H; *m*)
C14	124.59	5.10 (1H; *m*)
C15	131.47	
C16	25.89	1.60 (3H; *s*)
C17	16.21	1.60 (3H; *s*)
C18	16.22	1.60 (3H; *s*)
C19	16.49	1.68 (3H; *s*)
C20	17.88	1.68 (3H; *s*)

Table 1: ^{1}H and ^{13}C NMR, as well as by direct comparison with an authentic reference compound. The results of these analyses indicated that the compound was geranylgeraniol; ^{1}H-NMR ($CDCl_3$, 500.00 MHz).

[a]Chemical shift/ppm; [b]Chemical shift/ppm (number H; multiplicity; coupling constant) (Jondiko and Pattenden 1989).

Diterpenes	Rt (min) *	*m/z* (Relative intensity)
4,14-dihydroxy-dolastane-1[15],7,9-triene [1]	33.146	344 (6.75); 289 (5.32); 285 (6.61); 270 (2.02); 251 (6.27); 241 (23.54); 223 (10.78); 201 (14.96); 199 (11.50); 185 (17.19); 175 (17.45); 173 (15.69); 171 (15.27);163 (10.55); 165 (23.58); 162 (27.91); 159 (23.36); 149 (26.43); 145 (37.12); 136 (12.96); 133(25.69); 131 (22.46); 121 (32.40); 119 (27.48); 107 (23.74); 95 (20.17); 91 (37.88); 81 (21.47); 69 (26.57); 55 (38.51); 43 (100); 41 (38.09)
4-acetoxy-14-hydroxy-dolastane-1 [15],7,9-triene [2]	34.270	344 (11.11); 326 (20.20); 284 (7.66); 266 (22.28); 251 (34.15); 242 (35.66); 238 (17.25);227 (9.28); 223 (28.35); 209 (12.54); 199 (10.54); 181 (11.47); 157 (22.69); 145 (21.39);133 (45.66); 121 (28.88); 119 (34.47); 107 (24.68); 99(28.00); 95 (16.96); 91 (45.45); 79 (23.82); 55 (33.66); 43 (100); 41 (35.12)
4,7 diacetoxy-14-hydroxydolastane-1[15], 8-diene [3]	37.341	344 (8.82); 326 (10.21);301 (13.66);288 (12.46);269 (6.59); 266 (14.01);251 (17.60); 241 (23.63); 233 (19.01); 199 (10.48);185 (9.02);161 (10.54); 157 (15.83); 149 (28.46); 145 (26.20); 133 (33.80);121 (32.13); 119 (29.73); 107 (24.69); 95 (16.28); 91 (36.86); 79 (20.95);55 (28.06); 43 (100); 41 (27.21)
4-acetoxy-9,14-dihydroxy-1,9-dolastane-1,9-diene [4]	37.667	362 (1); 345 (1.56); 344 (6.51); 326 (8.1); 319 (10.14); 301 (18.58); 266 (10.26); 258 (8.63); 251 (15.24); 242 (21.95); 241 (45.21); 223 (19.37); 217 (19.53); 199 (28.28); 183 (10.28); 171 (10.38); 159 (10.64); 157 (19.90); 149 (19.69); 145 (20.27); 131 (17.31);123 (29.79); 119 (32.68); 107 (21.90); 105 (32.27); 93 (24.71); 79 (25.73); 43 (100); 41 (29.73)
Acetoxy Isolinearol [5]	38.353	350 (1.21); 335 (1.37); 318 (2.49); 247 (3.09); 233 (10.36); 219 (44.85);177 (8.12); 171 (10.79); 159 (40.09); 147 (12.27); 125 (30.65); 105 (18.31); 97 (8.04); 93 (10.33); 81 (11.90); 69 (22.59); 55 (25.89); 43 (100); 41 (23.41)

Table 2: Identified compound and main peaks with their relative intensity present in the mass spectra of diterpenes in *C.cervicornos* samples.

The appearance of new signals in the chromatograms over different reaction times, compared to the blanks, serves as another parameter of biosynthetic activity, indicating potential new reaction products. We detected four new signals at the 120-minute mark. Two signals at retention times 32.391 (M+ 281), 32.669 (M+ 280) are within the diterpenoid profile region in the chromatogram and their respective mass spectra show a similar fragmentation pattern to the other diterpenes. The identification and structural elucidation of these new signals will require other analytical techniques, which will be addressed in future studies.

Based on the evidence obtained from our experiments, we can argue that there may be a pool of enzymes in the aqueous extract of *C. cervicornis* that act by promoting rearrangements in the precursor, enabling processes of methylation and formation of double bonds and cyclization [23]. These enzymes can be considered cyclases because a method has been used to obtain cyclases in plants [22]. These enzymes can promote the cyclization of GGPP by diterpene cyclase, which can be initiated by ionization of the diphosphate ester, followed by hydride shifts, methyl migrations, Wagner-Meerwein

rearrangements, and other cyclizations of the resulting carbocation, such as internal deprotonations and deprotonation [32]. However, it is important to recall that diterpene cyclases can also cyclize GGPP by a mechanism initiated by protonation, sometimes coupled with a second cyclization by diphosphate ionization. The study and in-depth analysis of the cyclization mechanisms require several analytical techniques that were not the subject of our study.

Discussion

The identification of diterpenes in this aqueous extract is the first and important finding in our work because extraction with organic solvents is routinely used for the study of these molecules, which increases costs in the possible processes of obtaining them on an industrial scale and gives rise to an environmental impact. The identification of these diterpenes in the EA using mechanical extraction and aqueous buffer could serve as a starting point for exploring protocols and developing sustainable techniques for diterpene extraction.

The precursor used in the cyclase assays was analyzed by ^{1}H NMR and TLC, confirming the chemical shifts of GGOH in the isolated fractions; Notably, GGOH can be considered a precursor of diterpenes and has been identified in brown algae such as *Bifurcaria Bifurcata* [33]. Some studies report that GGOH is the major component of annatto seeds (1% of dry weight), making *B. orellana* the richest known source of this important terpene alcohol (C20) "all-E-geranylgeraniol" with up to 80% annatto crude extract [34] and which has also been reported to have important bioactivities [35]. In this and previous works [23,24], we propose that *B. Orellana* can be an interesting alternative for the recovery of this precursor, allowing its modification with enzymatic engineering not only to obtain important information about the cycle of diterpenes but also to open a sustainable pathway for the recovery of new diterpene molecules.

Cyclase enzymes function as biocatalysts, transforming substrates and accelerating their reactions. Factors such as temperature, pH, substrate concentration, and the presence of cofactors are crucial for optimal enzyme activity, as they affect the reaction rate [36]. One of the important cofactors for cyclases is the cofactor Mg^{2+}, for example; has been described several active sites of bacterial and fungal DTSs that exhibit several highly conserved motifs, involved in the binding of the cofactor Mg^{2+} that form a trinuclear cluster for the binding of the diphosphate unit of the substrate [17,37,38]. In plants it has been described how all monoterpene cyclases from gymnosperms use Mn^{2+} or Fe^{2+} as the necessary divalent cation and have an optimal alkaline pH [39], on the other hand, the cyclases of angiosperms prefer Mg^{2+} or Mn^{2+} do not require a monovalent cation, and have an optimal pH closer to neutrality [32,40]. Thus, the above indications show the importance of the cofactors $MgCl_2$ and $MnCl_2$ used in our reaction mixture. Considering our results obtained at a reaction time of 2 hours, we can propose $MgCl_2$ and $MnCl_2$ as an essential cofactor and its influence on the biosynthetic reaction of brown algae of the genus *Canistrocarpus*. Another important factor is the presence of the substrate GGPP in the biosynthesis reactions. In biosynthesis studies, GGPP is generally mentioned as a substrate for diterpenes [16,17,41]. To give attention that our GGPP was obtained according to the procedure of Keller and Thompson (1993) [26]. The possibility that the substrate GGOH and phosphate do not interact would reduce the likelihood of cycling processes, as the phosphate version of this precursor interacts specifically with diterpene cyclase enzymes. Studies aimed at monitoring and quantifying the conversion of GGOH to GGPP will be necessary to discuss such modifications of the substrates. Yet, the discussion revolved around the biosynthetic genesis of acyclic diterpenes from the precursor alcohol geranylgeraniol extracted from the brown alga *Bifurcaria bifurcata*, suggesting the potential derivation of our reaction products from alcoholic precursors [33]. It is also important to note that the often complicated cyclization mechanisms of DTSs have led research groups to extensively investigate DTSs for their potential to convert GGPP analogs into unnatural diterpenes [16] In this study, we showed that the factors tested in the reaction were effective for the performance of some enzymes present in the aqueous medium in contact with the GGOH precursor.

It should be noted that the use of PVP (1% w/v, Mr = 40,000) was a determinant of enzymatic activity after 2 hours, as the inclusion of these reducing agents can maximize enzymatic activity by adsorption of phenolic and lipophilic substances and attenuation of polyphenol oxidases [42]. Increased activity of extracted cyclases has been studied and reported in various plant parts [43]. Concentrations of polyphenolic compounds ranging from 0.1 to 1.4% have been reported for brown algae of the family Dictyotaceae, (*C. cervicornis*, *D. dichotoma*, *D. mertensii*, *Dictyopteris delicatula*, and *D. plagiogramma*) [44], although in low quantities (> 2% dry weight) these concentrations may increase under stress conditions like in the collection, transport and maceration of the seaweed to obtain the aqueous extract, which may affect enzymatic activity. Thus, the presence of PVP in the reaction mix of our protocol may have maximized the enzyme activity at 2h.

The best conditions for the enzymatic assays were the use of the buffer 50 mM Hepes (pH 7.8), 5 mM sodium ascorbate, 5 mM $Na_2S_2O_5$, 5 mM $MnCl_2$, 20 mM $MgCl_2$, and 10% (v/v) glycerol in a two-hour reaction at 30°C. Under these conditions and using geranyl diphosphate as substrate, it was possible to observe the activity of the cyclases; similar results have been obtained in other work on enzymatic activity in plants [45]. The protocol was also used to the determination of the activity of sesquiterpene synthases [46]. Comparable conditions were used in tests with diterpene cyclases specifically for *Vitis vinifera* [47], showing that the method developed for the catalytic extraction of active enzymes can provide us with an important tool for the study of diterpene synthase enzymes in seaweed.

In this study, it was possible to initiate the investigation of the biosynthetic pathway of diterpenes from the alga *Canistrocarpus cervicornis*. We investigated the reactivity between the precursor and the aqueous extract of *C. cervicornis* and an important influence of the cofactors $MgCl_2$ and $MnCl_2$. The new signals observed at different reaction times did not show the significant integration of the precursor and the obtained enzyme pool, several factors such as monitoring the conversion of GGOH to GGPP could be a critical point for investigation as they would provide quantitative data to confirm the use of the proposed precursor in the biogenic pathways of Dictyotaceae family algae and cycling processes. However, the consumption of GGOH after 2 h could indicate the possible influence of cyclase on substrate utilization.

This work pioneers the presentation of methods for enzymatic extraction and ideal conditions for enzyme activity of cyclases in algae. Our findings mark the initial steps toward a pathway that will facilitate the more efficient isolation of enzymes, characterization of diterpene synthase enzymes, and conducting biosynthesis assays with the target enzyme. New strategies and techniques such as genetic engineering

should be used to verify the enzymatic activity and characterize the terpene cyclases in *C. cervicornis.*

Highlights

- Enzymatic extraction and cyclase activity methods in Dictyotaceae algae.
- Reactivity between the GGPP precursor and the aqueous extract of *C. cervicornis.*
- Precursor consumption at 2 h indicates the influence of cyclase.
- Abundances of the type dolostane diterpenes increasing at 2h.

Funding

This research was funded by the CONSELHO NACIONAL DO DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO (CNPq) grant number 443930/2014-7 and Productivity Fellowship grant number 304070/2014-9 - VLT) and the FUNDAÇÃO DE AMPARO À PESQUISA CARLOS CHAGAS FILHO DO ESTADO DO RIO DE JANEIRO (FAPERJ), grant numbers E-26/110.205/2013, E-26/111.220/2014, E-26/211.069/2019, E-26/211.651/2021 and E-26/205.005/2022 (DC). CAPES doctoral scholarship (JMCO) and scientific initiation PIBIC/UFF/CNPq (TCS).

Acknowledgment

We would like to thank the Coordination for the Improvement of Higher Level Personnel (CAPES) for PhD (JO) and fellowship and Fundação de Amparo à Pesquisa Carlos Chagas Filho of the State of Rio de Janeiro (FAPERJ) for Training and Technical Qualification fellowship TCT- 5-(JO). We also thank the Scholarship Program of Scientific Initiation - PIBIC/UFF/CNPq (TCS) and express our gratitude to Dr. Roberto Campos Martins for his suggestions.

Conflicts of Interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Chen J, Li H, Zhao Z, Xia X, Li B, et al. (2018) Diterpenes from the Marine Algae of the Genus Dictyota. Mar Drugs 16: 159.
2. Bogaert KA, Delva S, Clerck O (2020) Concise review of the genus Dictyota JV Lamouroux. J Appl Phycol 32: 1521-1543.
3. Rushdi MI, Abdel-Rahman IAM, Attia EZ, Saber H, Saber AA, et al. (2022) The Biodiversity of the Genus *Dictyota*: Phytochemical and Pharmacological Natural Products Prospectives. Molecules 27: 672.
4. Oliveira AS, Cavalcanti DN, Bianco ÉM, Paula JC, Pereira RC, et al. (2008) Chemical composition of diterpenes from the brown alga *Canistrocarpus cervicornis* (Dictyotaceae, Phaeophyceae). Nat Prod Commun 39: 1934578X0800300913.
5. Campbell S, Murray J, Delgoda R, Gallimore W (2017) Two New Oxodolastane Diterpenes from the Jamaican Macroalga *Canistrocarpus cervicornis*. Mar Drugs 15: 150.
6. Vallim MA, Barbosa JE, Cavalcanti DN, Paula JC, Silva VAG, et al. (2010) In vitro antiviral activity of diterpenes isolates from the Brazilian brown alga *Canistrocarpus cervicornis*. J Med Plant Res 4: 2379-2382.
7. Cirne-Santos CC, Souza BC, Oliveira MC, Rabelo VW, Azevedo RC, et al. (2020) In vitro Studies on The Inhibition of Replication of Zika and Chikungunya Viruses by Dolastane Isolated from Seaweed *Canistrocarpus cervicornis*. Sci Rep 10: 8263.
8. Ribeiro MMJ, Sodero ACR, Diaz NC, Teixeira VL, Rodrigues CR, et al. (2022) Diterpenes isolated from *Canistrocarpus cervicornis* with virucidal activity against HIV-1: An in silico evaluation. Nat Prod Res 36: 5783-5787.
9. Figueiredo CS, Silva SMP, Abreu LS, Silva EF, Silva MS, et al. (2019) Dolastane diterpenes from *Canistrocarpus cervicornis* and their effects in modulation of drug resistance in *Staphylococcus aureu*s. Nat Prod Res 33: 3231-3239.
10. Camara RB, Costa LS, Fidelis GP, Nobre LT, Dantas-Santos N, et al. (2011) Heterofucans from the brown seaweed *Canistrocarpus cervicornis* with anticoagulant and antioxidant activities. Mar Drugs 9: 124-138.
11. Bianco ÉM, Rogers R, Teixeira VL, Pereira RC (2009) Antifoulant diterpenes produced by the brown seaweed *Canistrocarpus cervicornis*. J Appl Phycol 21: 341-346.
12. Moura LA, Bianco EM, Pereira RC, Teixeira VL, Fuly AL (2011) Anticoagulation and antiplatelet effects of a dolastane diterpene isolated from the marine brown alga *Canistrocarpus cervicornis*. J Thromb 31: 235-240.
13. Obando JMC, Santos TC, Bernardes M, Nascimento N, Villaça RC, et al. (2022) Chemical variation and analysis of diterpenes from seaweed *Dictyota menstrualis* under controlled conditions. Algal Res 62: 102637.
14. Obando JMC, Santos TC, Fortes RDR, Bernardes M, Nascimento N, et al. (2023) Influence of laboratory conditions on two species Dictyotaceae family (Phaeophyceae): Diterpenoid profile and physiological response. Front Mar Sci 10: 1150719.
15. Degenhardt J, Kollner TG, Gershenzon J (2009) Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry 70: 1621-1637.
16. Li H, Dickschat JS (2022) Diterpene Biosynthesis from Geranylgeranyl Diphosphate Analogues with Changed Reactivities Expands Skeletal Diversity. Angew Chem Int Ed Engl 61: e202211054.
17. Li H, Dickschat JS (2023) Enzymatic Synthesis of Diterpenoids from iso-GGPP III: A Geranylgeranyl Diphosphate Analog with Shifted Double Bond. Chem Eur J 30: e202303560.
18. Calegario G, Pollier J, Arendt P, de Oliveira LS, Thompson C, et al. (2016) Cloning and Functional Characterization of Cycloartenol Synthase from the Red Seaweed *Laurencia dendroidea*. PLoS One 11: e0165954.
19. Wei G, Jia Q, Chen X, Kollner TG, Bhattacharya D, et al. (2019) Terpene Biosynthesis in Red Algae Is Catalyzed by Microbial Type But Not Typical Plant Terpene Synthases. Plant Physiol 179: 382-390.
20. Kelecom A, Teixeira VL (1986) Diterpenes of marine brown algae of the family Dictyotaceae: their possible role as defense compounds and their use in chemotaxonomy. Sci Total Environ 58: 109-115.
21. Barton DHR, Mellows G, Widdowson DA, Wright JJ (1971) Biosynthesis of terpenes and steroids. Part IV. Specific hydride shifts in the biosynthesis of lanosterol and β-amyrin. J Chem Soc C: 1142-1148.
22. Lewinsohn E, Gijzen M, Savage TJ, Croteau R (1991) Defense mechanisms of conifers: relationship of monoterpene cyclase activity to anatomical specialization and oleoresin monoterpene content. Plant Physiol 96: 38-43.
23. Braga AC (2018) Aplicação de extratos enzimáticos para a produção de diterpenos de algas pardas [Thesis]. Niterói (RJ): Universidade Federal Fluminense. Portuguese.
24. Obando JMC (2018) Avaliação da formação de diterpenos em algas pardas marinhas do gênero *Dictyota* sob condições de campo e laboratório [Dissertation]. Niterói (RJ): Universidade Federal Fluminense. Portuguese.

25. Costa CLSD, Chaves MH (2005) Pigment extraction from *Bixa orellana* L. seeds: An alternative for experimental disciplines of organic chemistry. Quim Nova 28: 149-152.

26. Keller RK, Thompson R (1993) Rapid synthesis of isoprenoid diphosphates and their isolation in one step using either thin layer or flash chromatography. J Chromatogr 645: 161-167.

27. Lowry OH (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275.

28. Paula JC (2007) Química e morfologia de espécies brasileiras da tribo Dictyotaceae (Phaeophyceae) e avaliação dos diterpenos [Thesis]. Rio de Janeiro (RJ): Universidade Federal do Rio de Janeiro. Portuguese.

29. Araujo JM, Tappin MRR, Rocha Fortes R, Lopes-Filho EAP, Salgueiro F, et al. (2018) Chemodiversity of the brown algae *Canistrocarpus cervicornis* (Dictyotaceae, Phaeophyceae) in tropical and subtropical populations along the southwestern Atlantic coast of Brazil. J Appl Psychol 30: 611-618.

30. Ortiz-Ramírez FA, Aparecida-Vallim M, Cavalcanti DN, Teixeira VL (2013) Effects of the secondary metabolites from *Canistrocarpus cervicornis* (Dictyotales, Phaeophyceae) on fertilization and early development of the sea urchin *Lytechinus variegatus*. Lat Am J Aquat Res 41: 296-304.

31. Schmidt A, Wachtler B, Temp U, Krekling T, Seguin A, wt al. (2010) A bifunctional geranyl and geranylgeranyl diphosphate synthase is involved in terpene oleoresin formation in *Picea abies*. Plant Physiol 152: 639-655.

32. Davis EM, Croteau R (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. Biosynth 209: 53-95.

33. Munoz J, Culioli G, Köck M (2013) Linear diterpenes from the marine brown alga *Bifurcaria bifurcata*: A chemical perspective. Phytochem Rev 12: 407-424.

34. Basting RT, Abreu PMB, Sousa IMO, de Carvalho JE, Carvalho PRN, et al. (2021) *Bixa orellana* L. by-products' fractions from an industrial process: Antiproliferative activity on tumor cells and chemical profile. Nat Prod Res 35: 6106-6111.

35. Neto RNM, Coutinho GG, de Oliveira AR, Pontes DB, Ferreira RLPS, et al. (2020) Compounds isolated from *Bixa orellana*: Evidence-based advances to treat infectious diseases. Rev Colomb Quim 49: 581-601.

36. Vattekkatte A, Garms S, Brandt W, Boland W (2018) Enhanced structural diversity in terpenoid biosynthesis: Enzymes, substrates and cofactors. Org Biomol Chem 16: 348-362.

37. Rinkel J, Lauterbach L, Rabe P, Dickschat JS (2018) Two Diterpene Synthases for Spiroalbatene and Cembrene A from *Allokutzneria albata*. Angew Chem Int Ed Engl 57: 3238-3241.

38. Zhang P, Wu G, Heard SC, Niu C, Bell SA, et al. (2022) Identification and Characterization of a Cryptic Bifunctional Type I Diterpene Synthase Involved in Talaronoid Biosynthesis from a Marine-Derived Fungus. Org Lett 38: 7037-7041.

39. Bohlmann J, Steele CL, Croteau R (1997) Monoterpene synthases from grand fir (*Abies grandis*). cDNA isolation, characterization, and functional expression of myrcene synthase, (-)-(4S)-limonene synthase, and (-)-(1S,5S)-pinene synthase. J Biol Chem 272: 21784-21792.

40. Rajaonarivony JI, Gershenzon J, Croteau R (1992) Characterization and mechanism of (4S)-limonene synthase, a monoterpene cyclase from the glandular trichomes of peppermint (Mentha x piperita). Arch Biochem Biophys 96: 49-57.

41. Pemberton TA, Chen M, Harris GG, Chou WK, Duan L, et al. (2017) Exploring the Influence of Domain Architecture on the Catalytic Function of Diterpene Synthases. Biochemistry 56: 2010-2023.

42. Gershenzon J, McCaskill D, Rajaonarivony JI, Mihaliak C, Karp F, et al. (1992) Isolation of secretory cells from plant glandular trichomes and their use in biosynthetic studies of monoterpenes and other gland products. Anal Biochem 200: 130-138.

43. Chavez-Moctezuma MP, Lozoya-Gloria E (1996) Biosynthesis of the sesquiterpenic phytoalexin capsidiol in elicited root cultures of chili pepper (*Capsicum annuum*). Plant Cell Rep 15: 360-366.

44. Fleury BG, Kelecom A, Pereira RC, Teixeira VL (1994) Polyphenols, terpenes and sterols in Brazilian Dictyotales and Fucales (Phaeophyta). Bot Mar 37: 457-62.

45. Cao R, Zhang Y, Mann FM, Huang C, Mukkamala D, et al. (2010) Diterpene cyclases and the nature of the isoprene fold. Proteins; Struct Funct Bioinf 78: 2417-2432.

46. Pontin M, Bottini R, Burba JL, Piccoli P (2015) *Allium sativum* produces terpenes with fungistatic properties in response to infection with *Sclerotium cepivorum*. Phytochemistry 115: 152-60.

47. Escoriaza G, Sansberro P, Garcia-Lampasona S, Gatica M, Bottini R, et al. (2013) *In vitro* cultures of *Vitis vinifera* L. cv. Chardonnay synthesize the phytoalexin nerolidol upon infection by *Phaeoacremonium parasiticum*. Phytopathol Mediterr 52: 289-297.

HERALD

Advances In Industrial Biotechnology | ISSN: 2639-5665

Advances In Microbiology Research | ISSN: 2689-694X

Archives Of Surgery And Surgical Education | ISSN: 2689-3126

Archives Of Urology

Archives Of Zoological Studies | ISSN: 2640-7779

Current Trends Medical And Biological Engineering

International Journal Of Case Reports And Therapeutic Studies | ISSN: 2689-310X

Journal Of Addiction & Addictive Disorders | ISSN: 2578-7276

Journal Of Agronomy & Agricultural Science | ISSN: 2689-8292

Journal Of AIDS Clinical Research & STDs | ISSN: 2572-7370

Journal Of Alcoholism Drug Abuse & Substance Dependence | ISSN: 2572-9594

Journal Of Allergy Disorders & Therapy | ISSN: 2470-749X

Journal Of Alternative Complementary & Integrative Medicine | ISSN: 2470-7562

Journal Of Alzheimers & Neurodegenerative Diseases | ISSN: 2572-9608

Journal Of Anesthesia & Clinical Care | ISSN: 2378-8879

Journal Of Angiology & Vascular Surgery | ISSN: 2572-7397

Journal Of Animal Research & Veterinary Science | ISSN: 2639-3751

Journal Of Aquaculture & Fisheries | ISSN: 2576-5523

Journal Of Atmospheric & Earth Sciences | ISSN: 2689-8780

Journal Of Biotech Research & Biochemistry

Journal Of Brain & Neuroscience Research

Journal Of Cancer Biology & Treatment | ISSN: 2470-7546

Journal Of Cardiology Study & Research | ISSN: 2640-768X

Journal Of Cell Biology & Cell Metabolism | ISSN: 2381-1943

Journal Of Clinical Dermatology & Therapy | ISSN: 2378-8771

Journal Of Clinical Immunology & Immunotherapy | ISSN: 2378-8844

Journal Of Clinical Studies & Medical Case Reports | ISSN: 2378-8801

Journal Of Community Medicine & Public Health Care | ISSN: 2381-1978

Journal Of Cytology & Tissue Biology | ISSN: 2378-9107

Journal Of Dairy Research & Technology | ISSN: 2688-9315

Journal Of Dentistry Oral Health & Cosmesis | ISSN: 2473-6783

Journal Of Diabetes & Metabolic Disorders | ISSN: 2381-201X

Journal Of Emergency Medicine Trauma & Surgical Care | ISSN: 2378-8798

Journal Of Environmental Science Current Research | ISSN: 2643-5020

Journal Of Food Science & Nutrition | ISSN: 2470-1076

Journal Of Forensic Legal & Investigative Sciences | ISSN: 2473-733X

Journal Of Gastroenterology & Hepatology Research | ISSN: 2574-2566

Journal Of Genetics & Genomic Sciences | ISSN: 2574-2485

Journal Of Gerontology & Geriatric Medicine | ISSN: 2381-8662

Journal Of Hematology Blood Transfusion & Disorders | ISSN: 2572-2999

Journal Of Hospice & Palliative Medical Care

Journal Of Human Endocrinology | ISSN: 2572-9640

Journal Of Infectious & Non Infectious Diseases | ISSN: 2381-8654

Journal Of Internal Medicine & Primary Healthcare | ISSN: 2574-2493

Journal Of Light & Laser Current Trends

Journal Of Medicine Study & Research | ISSN: 2639-5657

Journal Of Modern Chemical Sciences

Journal Of Nanotechnology Nanomedicine & Nanobiotechnology | ISSN: 2381-2044

Journal Of Neonatology & Clinical Pediatrics | ISSN: 2378-878X

Journal Of Nephrology & Renal Therapy | ISSN: 2473-7313

Journal Of Non Invasive Vascular Investigation | ISSN: 2572-7400

Journal Of Nuclear Medicine Radiology & Radiation Therapy | ISSN: 2572-7419

Journal Of Obesity & Weight Loss | ISSN: 2473-7372

Journal Of Ophthalmology & Clinical Research | ISSN: 2378-8887

Journal Of Orthopedic Research & Physiotherapy | ISSN: 2381-2052

Journal Of Otolaryngology Head & Neck Surgery | ISSN: 2573-010X

Journal Of Pathology Clinical & Medical Research

Journal Of Pharmacology Pharmaceutics & Pharmacovigilance | ISSN: 2639-5649

Journal Of Physical Medicine Rehabilitation & Disabilities | ISSN: 2381-8670

Journal Of Plant Science Current Research | ISSN: 2639-3743

Journal Of Practical & Professional Nursing | ISSN: 2639-5681

Journal Of Protein Research & Bioinformatics

Journal Of Psychiatry Depression & Anxiety | ISSN: 2573-0150

Journal Of Pulmonary Medicine & Respiratory Research | ISSN: 2573-0177

Journal Of Reproductive Medicine Gynaecology & Obstetrics | ISSN: 2574-2574

Journal Of Stem Cells Research Development & Therapy | ISSN: 2381-2060

Journal Of Surgery Current Trends & Innovations | ISSN: 2578-7284

Journal Of Toxicology Current Research | ISSN: 2639-3735

Journal Of Translational Science And Research

Journal Of Vaccines Research & Vaccination | ISSN: 2573-0193

Journal Of Virology & Antivirals

Sports Medicine And Injury Care Journal | ISSN: 2689-8829

Trends In Anatomy & Physiology | ISSN: 2640-7752