ABSTRACT

The inherent value of nature is immeasurable. That being said, through bioprospection – the systematic search for functional products or processes from living organisms –, the oceans and marine life have emerged as a relevant source of biodiscoveries that hold significant economic worth. Particularly considering the pharmaceutical industry, an increasing number of natural molecules of marine origin have been making their way into pipelines and receiving approval for clinical use. Still, in its earliest days, this had become an extractivist practice, putting marine environments at risk and nearly driving species to extinction through over-collecting. While it is now well understood that exploration of the oceans’ living resources must withstand a sustainable agenda, thus, protecting the environment from unnatural genetic losses, it was the developments towards achieving more efficient bioprospective strategies and non-destructive but feasible means to assure product supply that pushed for the greatest advances in this field. Herein, we present our assessment of this story by telling it through the 20-year journey – and few detours – we took in the chemical and pharmacological study of the ascidian *Eudistoma vannamei*, a species endemic to the northeast coast of Brazil that retains novel natural products with remarkable modes of action. Indeed, ascidians figure among the most pharmacologically talented marine organisms, having yielded the active principles of three new anticancer drugs, one of which is being considered for repositioning towards the treatment of COVID-19. Finally, we argue that emphasizing the unceasing biotechnological potential of
marine biological diversity, exemplified herein by Brazilian ascidians, but certainly true worldwide for this and many other groups, would work in favor of raising awareness and supporting strategies to foster conservation of the oceans.

**Keywords:** blue biotechnology, marine biodiversity, ascidians, bioproducts, innovation.

**RESUMO**

O valor intrínseco da natureza é incomensurável. Dito isso, por meio da bioprospecção – a busca sistemática de produtos ou processos funcionais a partir de organismos vivos –, os oceanos e a vida marinha emergiram como uma fonte relevante de biodescobertas com significativo valor econômico. Particularmente considerando a indústria farmacêutica, um número crescente de moléculas naturais de origem marinha tem sido alvo de pesquisa e desenvolvimento e recebido aprovação para uso clínico. Ainda assim, em seus primeiros dias, isso se tornou uma prática extrativista, colocando os ambientes marinhos em risco e quase levando espécies à extinção por meio da coleta excessiva. Embora agora seja bem compreendido que a exploração dos recursos vivos dos oceanos deve suportar uma agenda sustentável, protegendo o meio ambiente de perdas genéticas não naturais, foi o desenvolvimento no sentido de alcançar estratégias bioprospectivas mais eficientes e meios não destrutivos, mas viáveis para garantir o suprimento do produto que impulsionou os maiores avanços nesse campo. Aqui, apresentamos essa história contando-a ao longo de uma jornada de 20 anos – e alguns desvios – que fizemos no estudo químico e farmacológico da ascídia Eudistoma vannamei, uma espécie endêmica do litoral nordeste do Brasil, que possui novos produtos naturais com modos de ação notáveis. De fato, as ascídias figuram entre os organismos marinhos mais talentosos farmacologicamente, tendo produzido os princípios ativos de três novos medicamentos anticâncer, um dos quais está sendo considerado para reposicionamento para o tratamento de COVID-19. Por fim, argumentamos que enfatizar o incessante potencial biotecnológico da diversidade biológica marinha, aqui exemplificado pelas ascídias brasileiras, mas certamente verdadeiro em todo o mundo para este e tantos outros grupos, funcionaria a favor da conscientização e apoio a estratégias de promoção da conservação dos oceanos.

**Palavras-chave:** biotecnologia azul, biodiversidade marinha, ascídias, bioproductos, inovação.

**INTRODUCTION**

Oceans hold inestimable and underexplored research opportunities. The innovation potential has been continuously evidenced as discoveries turn into products and knowledge about marine organisms and their biotechnological applications (Erwin; López-Legentil & Schuhmann, 2010; Paul et al., 2020). Over 31,000 compounds have been identified from marine organisms, and some are already used as medicine, food, cosmetics and other areas (Lyu et al., 2021). Despite being still in its infancy, the prospects for marine natural products are fast-growing within the blue biotechnology-related industries, with an estimated growth of USD 1.43 billion until 2025 (Market Watch, 2021).

In this realm, exploration of the oceans through bioprospection must be carried out in a sustainable manner and walk aside conservation policies in order to protect marine
FOSTERING CONSERVANCY THROUGH BIOPROSPECTION: THE PHARMACEUTICAL VALUE OF THE BRAZILIAN ASCIDIAN FAUNA

ecosystems from unnatural genetic losses, which are, in fact, the utmost source of biodiscoveries. Indeed, to act responsibly and protect the oceans and marine environments are part of the United Nations Sustainable Development Goal 14, which pledges to “conserve and sustainably use the oceans, seas and marine resources” (United Nations, 2021). Yet, bioprospection, and mostly that practiced with pharmaceutical purposes, imposed a predatory model in its earliest days. Due to the low yield of active compounds naturally present in the source organisms, marine invertebrates have been harvested in tons to enable the amounting of promising molecules for sequential pre-clinical and clinical studies. For example, the brown bryozoan *Bugula neritina* suffered massive collection out of its natural environment to achieve large-scale isolation of the natural product bryostatin 1. Approximately 13 tons of the organism yielded 13 g of the molecule (Koleck *et al.*, 1991) which would then be evaluated as an anticancer agent in the first clinical trial study conducted in 35 patients with Non-Hodgkin’s Lymphoma (Pluda; Cheson & Phillips, 1996). Similarly, tons of *Lissodendoryx* sp. and *Ecteinascidia turbinata* were collected to isolate grams of halichondrin B and trabectedin, respectively, allowing preclinical development, but also making clear that harvesting natural population was not a feasible option for commercialization of these drugs (Cuevas & Francesch, 2009; Jackson; Henderson & Phillips, 2009).

However, as conservation awareness arose, the experimental design of prospection of marine natural products projects evolved to subdue this problem. While chemical synthesis was failing to provide an efficient and economically viable pathway to scale-up the yields of desired marine molecules, alternative strategies have been put in place to overcome for these supply issues, and these include semi-synthesis starting from a highly available intermediate natural compound; direct extraction from maricultured organisms, e.g. the ascidian *Ecteinascidia turbinata* aiming obtenting of trabectedin; laboratory cultivation of free living bacteria or bacterial symbionts (when those are acknowledged as the producers of bioactive molecules previously isolated from a macroorganism); and even through genome manipulation of a microbial vector (Bauermeister *et al.*, 2018; Newman, 2018; Santos *et al.*, 2020; Wilke *et al.*, 2021).

While it has been estimated that over 90% of marine species still await to be discovered and formally described (Snelgrove, 2016), the World Register of Marine Species acknowledges, so far, about 240,000 species (WoRMS, 2021). Among those, there are approximately 3,000 known ascidian species and these organisms integrate the most prolific group of marine invertebrates regarding the production of bioactive compounds, along with sponges, cnidarians, and mollusks.

The anticancer chemotherapeutic agent trabectedin (named Yondelis® by PharmaMar) was isolated from the Caribbean ascidian *Ecteinascidia turbinata*. It is indicated for the treatment of patients with unresectable or metastatic liposarcoma or leiomyosarcoma and is under clinical studies for other cancers. Trabectedin well portrays a successful story in this field, by fulfilling an abiding therapeutic need and also generating huge economic income (Jimenez *et al.*, 2020). Following the approval of Yondelis®, lurbinectedin, a trabectedin derivative, and plitidepsin (also known as dihydridemnin B), obtained from the Mediterranean ascidian *Aplidium albicans*, were also approved as pharmaceuticals for anticancer treatment by PharmaMar as Zepzelca™ and Aplidin®, respectively (Jimenez *et al.*, 2020; Wilke *et al.*, 2021). It is worth mentioning that the latter compound has recently shown remarkable *in vitro* results in reducing the viral load in cells infected with SARS-CoV-2, the
virus responsible for launching the Covid-19 pandemic (White et al., 2021), and is currently undergoing phase III clinical trials to evaluate if plitidepsin can also produce relevant therapeutic benefits at patient level (U.S. National Library of Medicine – ClinicalTrials.gov – Identifier: NCT04784559).

Brazil holds nearly 20% of the biodiversity of the planet. The country is widely known for hosting a record number of vegetal species (Joly et al., 2019) and has been traditionally engaged in the systematic study of botanical sources of bioactive natural products (Pinto et al., 2002). Still, long before that, empirical approaches to diagnose functionalities of surrounding plants were already underway by native peoples (Pinto, 1995). Findings arisen from these bioprospective enterprises, even if they still fall short before the impressive Brazilian biodiversity, have greatly informed on the invaluable terrestrial natural product richness housed within these borders. On the other hand, bioprospection of marine organisms in Brazil has been in stage, rather steadily, merely since the 1990s. And only in rare cases have marine organisms been associated with some kind of popular use, pharmacological or otherwise. Therefore, a whole field is being built, literally, out of the blue, reaching for previous literature and scraping off clues for biotechnological potential from ecological observations.

Biological assessments of Brazilian marine natural products have been largely dedicated to exploring the anticancer potential of marine organisms (Wilke et al., 2021). In agreement with that, Wilke et al. (2021) described the vast pharmaceutical potential of ascidians from Brazil through anticancer compounds with unique mechanisms of action obtained from the yet mild number of studied species. Herein, we offer an appraisal on the importance of ascidians as a source of biotechnologically interesting molecules and, moreover, as cases by which we illustrate concepts and issues that evolve within the field of marine natural products. Furthermore, we examine, in detail and context, the journey of Eudistoma vannamei, an ascidian which has been a subject of our studies for the past 20 years. In this sense, the present review has two main purposes: to expose, through such narrative, the bioeconomical relevance of the country’s marine biological resources and to support bioprospection as means to widen awareness and funds for conservation of Brazilian marine environments.

**Ascidians as source of inspiration and information**

Chemical cues shape the function and structure of marine systems, where several molecules are produced and sensed by organisms as their main strategy to see, taste or hear the underwater world. Marine sessile animals are highly specialized in releasing chemical substances for communication (Hay, 2009; Puglisi et al., 2019), and ascidians, teamed with their associated microorganisms, figure among the most chemically prolific invertebrates (Dou & Dong, 2019; McCauley et al., 2020).

Asciidiacea is a class that includes colorful and morphologically diverse animals that have soft bodies involved by an outer tunic, a characteristic that is shared by other taxonomically related organisms and grants them the general cognomen ‘tunicate’ (Delsuc et al., 2006). Such morphological plasticity and multiple reproductive strategies, a consequence of their rapid evolution process and peculiar genetic combinations, has allowed ascidians to occupy a wide range of marine habitats (Holland, 2016; Tsagkogeorga et al., 2010). Phylogenetically, these organisms are strategically positioned between vertebrates and invertebrates, under the subphylum Urochordata and phylum Chordata.
Despite owning reduced genomes compared to other chordates, ascidians retain many of their conserved genes, suggesting they are chordates closest ancestors (Delsuc et al., 2006; Fodor et al., 2021). Nevertheless, ascidians drop most of their chordate characteristics, i.e., a notochord during their free-swimming larval stage, by simplifying their bodies to adapt to adulthood, and exist for most of their life as sessile, filter-feeding organisms (Holland, 2016; Karaiskou et al., 2015).

The chemical richness of ascidians has been investigated since the 1960s, and this feature may also be attributed to their rapid evolution and phylogenetic position (Holland, 2016; Tsagkogeorga et al., 2010). As soft bodied, sessil, benthic animals, ascidians bear strategies to survive in an environment with high ecological pressures. The tunic, which is mainly composed of a self-elaborated unique cellulosic matrix, operates as a first defense against predation and infections. Ascidians have also developed a complex and well-developed immune system to fight pathogens, which functions with specialized cells such as phagocytes and other hemocytes containing cytotoxic components (Franchi & Ballarin, 2017; Satake et al., 2019). In addition, the production of chemical compounds by these organisms figure as another important ability to defend themselves from environmental injuries and other ecological interactions, such as larval settlement (Palanisamy; Rajendran & Marino, 2017; Puglisi et al., 2019).

Around 1,200 natural molecules have been, so far, identified from ascidians, while the last two decades yielded most of the described metabolites. Alkaloids, polyketides and peptides cover the main classes of compounds isolated from ascidians, many of which display unique structures rarely found in terrestrial sources (Dou & Dong, 2019; Palanisamy; Rajendran & Marino, 2017; Ramesh et al., 2021; Watters, 2018). As expected for other sessile organisms, the presence of associated microbes and symbionts within tunicates seem to be fundamental for the rich and diversified arsenal of natural products and bioactive compounds (Kwan et al., 2014; Simmons et al., 2008). Indeed, a number of substances initially isolated from ascidians were then shown to be produced by guest-microorganisms (Chen; Fu & Wang, 2017; Schmidt, 2015).

The production of bioactive compounds by ascidians and their associated microbiota can be understood from the perspective of the holobiont concept (Simon et al., 2019). In a holobiont system, the invertebrate provides a favorable internal environment to harbor a diverse assemblage of microorganisms which, in turn, provides essential benefits to the host, operating as a single unit (McFall-Ngai et al., 2013; Simon et al., 2019). Ascidians have developed intrinsic relationships with bacteria and other microorganisms. The filter-feeding behavior of ascidians provides an intense and constant exchange of microorganisms with the external environment, favoring the occurrence of an unexpectedly high diversity of microbes. Furthermore, the tunic is an attractive space for the recruitment of such microbiota, since it provides a shelter environment and nutrients availability (Bauermeister et al., 2019). On the other hand, associated-microorganisms can benefit their hosts as a direct food source or, indirectly, by fixating carbon and nitrogen. However, their involvement in providing natural compounds with biological properties certainly figures among the most-welcome functions (Dou & Dong, 2019; McFall-Ngai et al., 2013; Newman & Hill, 2006).

Using different culture-dependent and independent methods, a rich diversity of proteobacteria, fungi, cyanobacteria, and other microbes have been found to live in association with ascidians in both intra and extracellular spaces. A positive correlation of species-specific and tissues-specific occurrences was also found, emphasizing the
particularity of this collaboration (Chen; Fu & Wang, 2017; Chen et al., 2018). For example, the obligate symbiotic cyanobacteria Prochloron sp., found in the tunic of some ascidians from the Didemnid family, provide important substances for host photoprotection, where the color pigmentation produced by the microorganism follow ascidian photoadaptation in order to prevent damages caused by UV radiation and active oxygen forms (Hirose et al., 2006; Lesser & Stochaj, 1990). Moreover, to investigate the chemical variation in a set of specimens of the ascidian Lissoclinum patella, Kwan and collaborators found a subset of three divergent populations that were grouped according to their phylogeny, chemical profile and bacterial associations (Kwan et al., 2014). Of special interest, the work showed that chemical variation in symbiotic systems can be controlled by the host yet uncovered cryptic speciation, highlighting how a unique and serendipitous discovery of exclusive natural compounds could be found by surveying individual ascidian colonies.

The rich chemical diversity found in tunicates surpassed the studies of ecological functions and soon reached the benches of pharmacological laboratories. Compounds isolated from ascidians and associated microbes have been extensively investigated about their potential as new drug candidates to treat many diseases. Unsurprisingly, this prolific chemical source is reflected in substances with biomedical activities such as anticancer, antimicrobial, antiparasitic, antiviral and anti-inflammatory, both in vitro and in vivo (Dou & Dong, 2019; Luan et al., 2012; Ramesh et al., 2021; Watters, 2018). So far, three molecules originally found in ascidians have been approved for use as anticancer chemotherapy by international regulatory agencies, namely: trabectedin (also known by ecteinascidin 743 or ET-743; Yondelis®), lurbinectedin (Zepzelca™), which is analogous to trabectedin, and plitidepsin (also known as dehydrodidemnin B; Aplidin®) (Dyshlovoy & Honecker, 2020; Jimenez et al., 2020). Figure 1 addresses some significant events in the course of discovery and development of these ascidian-derived drugs.

The development of Yondelis® brought important lessons for the research and development of marine natural products as medicines, as indicated in the timeline in Figure 1 and extensively revised and cited in other works (D’Incalci & Galmarini, 2010; Jimenez et al., 2020; Van Kesteren et al., 2003). In 1969, biological activity for extracts of E. turbinata was reported for the first time, when researchers observed potent antitumor and immunosuppressive properties in vivo (Sigel et al., 1970). Nevertheless, the lack of sensitive techniques available at that time delayed isolation and structural characterization of trabectedin in 20 years (Rinehart et al., 1990). Moving on, trabectedin endured a long and dramatic path towards supplying enough material to contemplate all stages of clinical testing and commercial development, including a massive but unsuccessful effort at ascidian aquaculture and many attempts at total synthesis of this complex alkaloid. Finally, the industrial production of Yondelis® was made viable through a semisynthesis process using cyanosafracin B as a starting material, an antibiotic easily produced by the bacteria Pseudomonas fluorescens through large-scale fermentation, followed by a few synthetic steps (Cuevas et al., 2000; Cuevas & Francesch, 2009).

The investigation of trabectedin’s natural occurrence in E. turbinata has contributed significantly to the entire process of Yondelis® development and brought to light an interesting perspective within the holobiont concept. Firstly, structural similarities between trabectedin and bacterial natural products, i.e., cyanosafracin B and other saframycins, suggest a prokaryotic origin to the compound (Manzanares et al., 2001; Piel, 2006). Comparative analysis on bacterial diversity of E. turbinata collected at two distant locations
identified the proteobacteria “Candidatus Endoecteinascidia frumentensis” as a specific and persistent bacteria associated within the ascidian (D’Incalci & Galmarini, 2010; Pérez-Matos; Rosado & Govind, 2007).

A deeper look into the genetics of “Ca. E. frumentensis” revealed the presence of biosynthetic genes and enzymes required for trabectedin biosynthesis. However, a number of other key genes involved in the entire compound production were still missing (Schofield et al., 2015). Interestingly, a previous work using metaproteomic analysis of “Ca. E. frumentensis” revealed the candidate bacteria owned a reduced genome, lacking genes involved in their primary metabolism, such as peptidoglycan and lipid A biosynthesis or early glucose metabolism (Rath et al., 2011; Schofield et al., 2015). Taken together, the information above strongly supports the hypothesis that “Ca. E. frumentensis” and E. turbinata operate as a holobiont system, in which the bacteria rely on the host animal for survival, possibly through the use of essential metabolites, while the microbe provides the ascidian with precursors for the production of important secondary metabolites, such as trabectedin (Morita & Schmidt, 2018). Since the complete set of genes for biosynthesis of trabectedin remains to be identified, scientists believe that other microbes could be involved in these final steps or that interaction between the symbiotic proteobacteria and the host ascidian is required to conclude the process (Dou & Dong, 2019; Pérez-Matos; Rosado & Govind, 2007; Rath et al., 2011).

Figure 1 – Timeline highlighting significant events and important findings throughout the discovery and development of anticancer drugs originated from active principles sourced from ascidians. Abbreviations: ATGA – Australian Therapeutics Goods Administration; EMA – European Medicine Agency; FDA – U.S Food and Drug Administration; MNP – Marine Natural Product.
A second ascidian-derived star molecule is plitidepsin (Aplidin®), also known as dehydrodidemnin B, which represents the long-awaited arrival of didemnins in the clinic. For now, it has been granted approval only by the Australian regulatory agency in 2018, for the treatment of refractory multiple myeloma in association with dexamethasone (Jimenez et al., 2020). Isolated from Aplidium albicans, the cyclic depsipeptide plitidepsin was reported for its potent in vivo and in vitro antiproliferative activity in 1996 (Urdiales et al., 1996) and entered clinical trials two years later (Alonso-Álvarez et al., 2017). Total chemical synthesis ensured supply of plitidepsin during the 22 year long clinical development period and subsequent commercialization (Jimenez et al., 2020; Jou et al., 1997). Importantly, the development process of Aplidin® trailed in the tracks previously opened by didemnin B, a structurally related molecule isolated from the extracts of the Caribbean ascidian Trididemnum solidum, as noted in Figure 1. Didemnin B began clinical testing in 1986 as the first marine natural product to go into this stage, over a decade before plitidepsin reached this mark (Alonso-Álvarez et al., 2017; Lee et al., 2012). Mechanism of action studies with didemnin B faced some challenges in correlating the potent antiproliferative activity with the ability to inhibit protein synthesis (Jimenez et al., 2020). Once the role of elongation factor 1A2 (eEF1A2) in eukaryotic protein synthesis was better resolved, in addition to the understanding the oncogenic properties thereof – usually overexpressed in several types of cancer, including multiple myeloma –, it was possible to attribute that both didemnin B and plitidepsin used this protein as their primary molecular target (Ahuja et al., 2000; Alonso-Álvarez et al., 2017). However, didemnin B had its clinical development discontinued due to its low efficacy and a significant toxicity issue, which included neuromuscular effects and cardiotoxicity (Jimenez et al., 2020; Lee et al., 2012).

As trabectedin, didemnin B – and, hence, plitidepsin – is also considered a metabolite of probable microbial biosynthetic origin, since this and other didemnins were isolated from free-living α-proteobacteria Tistrella mobilis and Tistrella bauzanensis, recovered from the water column and marine sediments (Tsukimoto et al., 2011; Xu et al., 2012). The study further identified the didemnin B biosynthetic gene cluster from the genome of the T. mobilis strain, which suggests that a possible microbial association would be implicated in the biosynthesis of didemnins previously isolated from marine invertebrates (Xu et al., 2012).

**Pharmaceutical potential of Brazilian ascidians**

Ascidians appear amongst the most assessed and promising groups of organisms for chemically interesting and bioactive molecules in publications by Brazilian research groups (Figure 2). Since the earliest expeditions, investigative efforts have accessed species belonging to at least 22 different genera, apart from dozens of unidentified ascidians. Initially, bioprospection studies of Brazilian ascidians addressed specimens collected on the southeast coast and islands, which is a region harboring an ascidian fauna recognized by its richness and endemcity within the Atlantic Ocean (Moreno; Faria & Rocha, 2014; Wilke et al., 2021).

A widespread screening study by Seleghim et al. (2007) can be used to illustrate this statement. This publication reports the assessment of 349 extracts obtained from marine invertebrates collected between 1995 and 2002 along the coast of Bahia, Rio de Janeiro and São Paulo states in search of bioactive samples. Among the 99 ascidian tested, 20 of them were yet unidentified species, while 60% of the extracts displayed activity in at least one of the five assays employed, mainly antibacterial and cytotoxic. This study highlighted the
genera *Didemnum*, *Polysyncraton*, *Clavelina*, *Herdmania*, *Aplidium*, *Microcosmus*, *Eusynstyella*, *Botrylloides* and *Symplegma* as the leading producers of bioactive compounds.

Prado *et al.* (2004) conducted a screening of 40 extracts derived from marine invertebrates, of which 16 were obtained from ascidians collected on the coast of São Paulo and Rio de Janeiro states. *Cystodytes dellechiajei* and *Didemnum* sp. produced two of the seven most cytotoxic extracts, while the first also disrupted the microtubule structure in cultured cells (Prado *et al.*, 2004). The extract of *C. dellechiajei* was further assessed by Torres *et al.* (2002) and yielded two cytotoxic pyridoacridine alkaloids with novel ring systems, designated sebastianines A and B. Both compounds displayed cytotoxicity against p53 or p21 knockout colon cancer cells HCT-116, as well as to the respective parental cells (Torres *et al.*, 2002).

Two unique poliheteroaromatic alkaloids, granulatimide and isogranulatimide, along with a new didemin derivative, dideminin E, and the known didemmins A and D were isolated from *Didemnum granulatum*, another ascidian from the south and southeast coast of Brazil, collected from two sites in São Paulo State – Araçá Beach and São Sebastião Channel – and one in Santa Catarina – at the Arvoredo Marine Biological Reserve. Both alkaloids induced prominent inhibition of the G2-checkpoint in a breast cancer cell line (Berlinick *et al.*, 1998; Roberge *et al.*, 1998) and also repressed the activity of Chk1 and Cdk1, kinases involved in the G2-M transition (Jiang *et al.*, 2004). Furthermore, Britton and collaborators (2001) reported the isolation of a third poliheteroaromatic alkaloid, 6-bromogranulatimide, along with dideminin C, as minor compounds of the extract of *D. granulatum*. Interestingly, a confocal microscopic examination of the ascidian’s tissues revealed the accumulation of granulatimide and isogranulatimide in bladder cells in the
ascidian upper tunic (Britton et al., 2001). Seeing that, these alkaloids may play a protective role against predators and confer photoprotection to the colony, as these molecules also have intense radiation absorption within the visible UV range (Seleghim et al., 2007).

Expanding the chemical space of metabolites from Brazilian ascidians, it is with mentioning the unique glycosaminoglycans (GAG) obtained from two species of solitary ascidians collected on the coast of Rio de Janeiro, Styela plicata and Phallusia nigra. These polysaccharides, dermatan sulfates (DS), were extracted from the ascidian species and are composed, respectively, by 2,4-O-sulfated and 2,6-O-sulfated disaccharide units. The ascidians DSs showed anticoagulant, antithrombotic, anti-inflammatory and antimetastatic activities (Kozlowski; Pavão & Borsig, 2011; Pavão et al., 1995). A study by Kozlowski and collaborators (2011) demonstrated that the biological activities of ascidian DS are specifically due to inhibition of P-selectin-mediated interactions. P-selectin is a glycoprotein involved in intercellular adhesion processes and contributes to pathological conditions, such as inflammatory tissue injury, pathologic thrombosis and metastasis (Chen & Geng, 2006). Initially, the authors observed that, in contrast to mammalian DS, both ascidians DSs were able to potently inhibit binding of human colon carcinoma cells to immobilized P-selectin. The antimitastatic effect of ascidian DSs was observed in mouse colon carcinoma cells stably expressing GFP (MC-38GFP) and, less efficiently, in mouse melanoma cells (B16-BL6). Additionally, both ascidians DSs prominently reduced platelet deposition and thrombus size, as well as peritonitis and infiltration of inflammatory cells in mouse models (Kozlowski; Pavão & Borsig, 2011).

In another study from the same research group, Abreu et al. (2019) isolated another distinct GAG from the viscera of P. nigra, a heparan sulfate (HS) particularly enriched in 2-sulfated β-glucuronic acid units. The ascidian HS also inhibited adhesion of tumor cells onto immobilized P-selectin at an 11-fold potency compared to mammalian heparin. As it displayed an inexpressive anticoagulant activity, this HS from P. nigra figures as a potential therapeutic alternative to mammalian heparin for treatment of inflammation and tumor metastasis (Abreu et al., 2019). Heparin is an ancient but extremely useful anticoagulant/antithrombotic drug from the sulfated glycosaminoglycans family to which anti-inflammatory and antimitastatic properties were also also assigned (Abreu et al., 2019; Kozlowski; Pavão & Borsig, 2011). It is worth mentioning that heparin therapy has been recently considered a potential supportive treatment to alleviate systemic symptoms of COVID-19 and also for its anti-SARS-CoV-2 activity, although considerable security concerns have been listed, including risks of excessive bleeding (Shi et al., 2021; Yu et al., 2021). In this context, heparin analogues that lack anticoagulant activity, such as ascidians heparans may be explored as alternatives for treating COVID-19 patients without the risk of bleeding events (Kwon et al., 2020). The relative abundance of ascidian glycans in ascidian tissue and the technical feasibility of obtaining them are contrasted by the challenge of supplying these compounds, which would depend on cultivating the species for large-scale production (Abreu et al., 2019).

Reaching the coast of the northeast region of Brazil, Vervoort and collaborators (2000) explored the chemistry of a Didemnum sp. collected in a shallow-water reef in Tamandaré, Pernambuco State. Following the detection of potent cytotoxic activity in vitro, bioassay-guided fractionation of Didemnum sp. extract led to the isolation of two new cytotoxic depsipeptides, tamandarins A and B, which are structurally related to didemmins. Tamandarin A showed a highly cytotoxic activity profile in a colony formation assay.
FOSTERING CONSERVANCY THROUGH BIOPROSPECTION: THE PHARMACEUTICAL VALUE OF THE BRAZILIAN ASCIDIAN FAUNA

against cancer cell lines at nanomolar concentrations and slightly more cytotoxic than didemnin B (Vervoort et al., 2000). The authors pointed out that tamandarin A was able to inhibit protein synthesis in a mammalian cell-free system (Vervoort et al., 2000), like didemnin B. In fact, due to their structural similarities, it would be expected that didemnins and tamandarins would also share a mode of action, and structure-activity studies have corroborated this premise (Ahuja et al., 2000; Liang et al., 2001).

A cytotoxicity screen published by our group (Jimenez et al., 2003) assessed the hydromethanolic extracts obtained from 10 ascidian collected along the coast of Ceará, in the northeastern region of Brazil. This publication came out at a time when little to no information had yet been generated concerning the description of the ascidian fauna or the bioprospective potential of the organisms from this region. According to data of the Web of Science Core Collection and other sources, this article has been cited in major systematic reviews of the field of marine natural products (Mayer & Gustafson, 2006). Within our then recently established research group, this debut work in the field of marine bioprospection befall a great impact and has been guiding project choices to this day.

Jimenez et al. (2003) revealed that, besides a great degree of endemism occurring among the selected species, 6 out of the 10 studied ascidian extracts presented cytotoxicity in at least one bioassay (Jimenez et al., 2003). Didemnum psammathodes, a species well distributed within the tropics but not ever addressed for its chemical components or bioactivity, displayed antimitotic properties in sea urchin eggs. Follow-up studies revealed the presence of free nucleosides, sterols, alcohols, methyl esters, glyceryl ethers and fatty acids in this extract (Takeara et al., 2007). A mixture of the methyl esters was cytotoxic against leukemia cell lines, inducing inhibition of DNA synthesis and cell death (Takeara et al., 2008). Furthermore, Euherdmania sp., a genus represented by only 13 species which had not yet been investigated for their chemistry and biological activities, displayed significant cytotoxicity against cancer cell lines. Feeding on these findings, a recent publication from our group examined the culturable microbiota associated with this species and found a promising source of bioactive compounds therein: an actinobacteria identified as Streptomyces sp. BRA346 was shown to produce a set of α’-β’-epoxyketones compounds related to eponemycin, including dihydroeponemycin (Furtado et al., 2021). Compounds in this class are potent modulators of proteasome catalytic activity and, indeed, have served as a structural scaffold for the anticancer drug carfilzomib (Kyprolis®), a selective proteasome inhibitor used to treat relapsed multiple myeloma. The study also revealed antiglioma potential for dihydroeponemycin and for a BRA346 fraction-containing a mixture of α’-β’-epoxyketones, further relating that to their antiproteasome activity and modulation of the unfolded protein response (Furtado, 2021).

Eudistoma vannamei: one ascidian, many opportunities

Among the 10 ascidians assessed in the bioactivity screen carried out by our group (Jimenez et al., 2003), Eudistoma vannamei, an endemic species to the northeast coast of Brazil and most abundant ascidian for the State of Ceará (Lotufo & Silva, 2006), arose as the most motivating species to further studies and remains, to this day, a relevant matter of our research group. Figure 3 outlines the studies undertaken with E. vannamei extract as well as those that investigated its associated microbiota. The extract displayed high toxicity against brine shrimp, antimitotic effect in sea urchin eggs and, remarkably, potent cytotoxicity in cancer cell lines (Jimenez et al., 2003). Therefore, by then, the main goals
were to explore the chemical diversity in this extract, isolate and elucidate the active principles and characterize their anticancer potential.

Various studies have been carried out aiming to identify chemical components of *E. vannamei*. Novel and unusual adenine alkaloid derivatives, 9-[N-(leucyl)-isoleucyl]-adenine and 8-hydroxy-8-isopentyl-7,8-dihydroadenine, and a phenylalanine derivative N-[N-(leucyl)-isoleucyl]phenethylamine were isolated from a methanolic extract (Pimenta et al., 2014). Additionally, a more apolar fraction allowed the identification of cholesterol, sitosterol and stigmasterol through a gas chromatography-mass spectrometry platform (Takeara et al., 2015). The presence of typical phytosterols in the animal’s extract was suggested to be acquired through their diet, which may include phytoplanktonic organisms. Figure 4 illustrates the diversity of chemical compounds isolated from *E. vannamei* and associated microorganisms.

Figure 3 – Flowchart outlining the different investigations and study designs carried out by our research group following the bioactivity screening of ascidians from Ceará State. Species selection for further projects were motivated by the cytotoxicity results displayed by their extracts, and included *Didemnum pasammatodes*, *Euherdmania* sp. and, remarkably, *Eudistoma vannamei*. The main driving questions posed by each study are depicted in blue circles, while research strategies employed to seek the respective answers are given in yellow rectangles.

A tandem mass spectrometry method, developed to rapidly screen ascidian extracts for nucleosides, identified four purine derivatives, those being adenine, 2′-deoxyadenosine, deoxyguanosine and 2′-deoxyguanosine (Takeara et al., 2007). Three pyrimidine nucleosides were further isolated from a methanolic extract and elucidated as thymidine, uridine and 2′-deoxyuridine (Takeara et al., 2015). Although these compounds were not subjected to bioassays, nucleosides and their derivatives are known to be toxic in cell, parasites and virus models for their ability to be inserted into DNA or RNA during replication, but to not allow elongation of the respective strand, thus blocking nucleic acid synthesis (Huang et al., 2014). Indeed, nucleosides from a marine sponge, *Tectitethya crypta* (Bergmann & Feeney, 1951), served as prototypes for the development of the first drugs of marine origin, cytarabine and vidarabine, launched in...
the market in 1969 and 1976, respectively, for the treatments of leukemias and virus infection (Jimenez; Wilke & Costa-Lotufo, 2018).

Figure 4 – Chemical diversity identified in *Eudistoma vannamei* and associated microorganisms

To pursue investigation of the outstanding anticancer activity, the ascidian was recollected, re-extracted and the crude extract was subjected to a bioassay-guided fractionation. This following study revealed that enriched fractions of intermediate polarity, which seized, indeed, similar chemical composition, were highly cytotoxic to human tumor cells, with IC₅₀ varying between 0.08 and 0.35 μg/mL – values that represented a potency up to 100 times that of the crude extract according to the cell line (Takeara et al., 2008). By these results, one of two hypotheses could be presumed: the active compounds were present in high yields, or the active compounds were extremely potent. A human leukemia cell model was chosen to address the mode of action of these fractions. Even if that varied slightly in potency, most fractions induced comparable results: while a lower concentration (0.1 μg/mL) induced a cytostatic effect, a concentration 10 times higher was sufficient to trigger cell death, mostly by apoptosis.

At this point, even with an enriched fraction in hand and phenotypical leads on the mode of action, it was not yet possible to single out the metabolite nor the chemical class responsible for the bioactivity. To advance and particularize the biological assessment with a sample that still retained some level of complexity was assumed a poor idea, as it could generate confusing and misleading results. However, more material was needed to endure a second round of bioassay-guided fractionation, and a new collection was carried out. This new extract was subjected to a cytotoxicity-guided fractionation, and, this time, all the way to obtaining 5 mg of an exceptionally potent mixture of two novel staurosporine derivatives, 2-hydroxy-7-oxo-staurosporine and 3-hydroxy-7-oxo-staurosporine (Jimenez et al., 2012). The staurosporines are highly cytotoxic indole-carbazole alkaloids that target protein kinases, which are cellular enzymes involved in many essential functions, such as...
metabolism, cell cycle and cytoskeletal arrangement (Lawrie et al., 1997; Manning et al., 2002). The parental molecule in this group, staurosporine (AM-2282), was obtained from soil actinobacteria Streptomyces staurosporeus (Omura et al., 1977) which later served as the precursor molecule for the semi-synthetic drug midostaurin (Rydapt®), a tyrosine kinase inhibitor approved in 2017 for the treatment of acute myeloid leukemia (AML) and other disorders (Stone et al., 2017).

Studies have reported the occurrence of staurosporines in extracts obtained from other Eudistoma species, notably in the Micronesian ascidian E. toealensis. Schupp et al. (1999, 2001) and Schupp, Proksch and Wray (2000) described the isolation and nM-cytotoxicity of 12 staurosporines obtained from the aforementioned ascidian and from its predatory flatworm Pseudoceros sp., hinting a bioaccumulation course of cytotoxic compounds by the flatworms through their diet. Later, through a molecular taxonomic approach, that group revealed E. toealensis to host an exceptionally high microbial diversity, including the occurrence of known staurosporine-producing actinobacteria genera, such as Verrucosispora and Salinispora, further suggesting a microbial origin of the compounds previously isolated from the host tissues (Steinert; Taylor & Schupp, 2015). The novel derivatives obtained from E. vannamei were about 10 times more cytotoxic than a staurosporine standard, and, also, displayed IC50 values in the nM-range, between 10 and 144 nM, against a tumor cell line panel of different histological origins. Remarkably, a non-tumor cell model showed a 5 to 70-fold resistance to the derivatives. Furthermore, at a lower concentration, the derivatives induced a cytostatic effect in leukemia cells, materialized by a sustained G2 cell cycle arrest, while a higher concentration prompted cells to DNA damage and apoptosis (Jimenez, 2009; Jimenez et al., 2012). The signaling pathways involved in cell cycle blockage induced by the staurosporine derivatives are represented in Figure 5.

For the first bioassay-guided fractionation, a little over 1 kg of ascidian biomass was collected from the environment and processed (Jimenez et al., 2008). To see through the second fractionation, which indeed led to isolation and structure elucidation of small amounts of the highly bioactive compounds, almost 9 kg of biomass was collected over several months and resorting to different sites of occurrence of the organism (Jimenez et al., 2012). Even though the population of E. vannamei was completely restored between spaced-out expeditions, harvesting increasingly large amounts of organisms from their natural habitat is not a sustainable practice, much less could this be endured long term.

There is a saying that goes around in the field of bioprospection: nature can provide for the search, but not the supply (Beattie et al., 2011). Nonetheless, supply has been the Achilles heel of natural product research and even more so when considering marine natural products. Chemical synthesis, the preferred means for industrial supply of natural compounds, has posed many challenges when attempted with structurally complex champion molecules of marine origin, especially those used as pharmaceuticals (Costa-Lotufo et al., 2009; Jimenez et al., 2020). Therefore, alternative routes have been proposed and optimized, streaming reliable, ecologically sane and economically viable processes of marine molecules into the market.

Led by the background knowledge on the origin of natural staurosporines, a class of compounds typically produced by microorganisms, efforts were turned to the search of culturable microbial producers associated to E. vannamei of those and, possibly, other bioactive compounds. In this context, 11 fungal strains were isolated, grown in liquid
FOSTERING CONSERVANCY THROUGH BIOPROSPECTION: THE PHARMACEUTICAL VALUE OF THE BRASILIAN ASCIDIAN FAUNA

media and extracted with organic solvents, yielding a mycelium and a broth extract for each strain. Over half of the tested samples displayed cytotoxicity, while broth extracts were generally more active than mycelia (Montenegro et al., 2012). An isolate identified as *Aspergillus* sp. EV10 yielded three isocoumarins and penicilic acid. The later compound displayed moderate *in vitro* cytotoxicity against tumor cells (Montenegro et al., 2012).

Alongside the efforts for isolation of fungi, bacteria associated with *E. vannamei* were also recovered, purified and assayed for cytotoxicity. In this setting, most efforts were directed to the recovery of Actinobacteria, a class of bacteria well and widely recognized for owning a prolific secondary metabolism which have sourced thousands of bioactive compounds, many of which have made the active principles of many drugs (Bérdy, 2005; Imhoff; Labes & Wiese, 2011). Additionally, actinobacteria are the best-known producers of staurosporines (Ōmura; Asami & Crump, 2018), the class of molecules that sparked the ascidian’s microbial bioprospection in the first place. An initial round of bacterial isolation recovered 84 strains, out of which 17 were assessed for cytotoxicity. One strain, *Streptomyces* sp. BRA010 (previously and therein codified as EVA01063) stood out for the potent effect of the extract against tumor cell growth across a three-cell line panel, with IC\(_{50}\) values near 1 μg/mL. Indeed, staurosporine was identified, through an analytical chemistry strategy, as a component in this extract and, thus, cytotoxicity of this extract could be attributed, at least in part, to the presence of this compound. However, the novel derivatives 2-hydroxy-7-oxo-staurosporine and 3-hydroxy-7-oxo-staurosporine, isolated from *E. vannamei* could not be identified in the bacterial extract (Andréeo et al., 2012).
Moving on, through a different study approach to the strain *Streptomyces* sp. BRA010, Abreu *et al.* (2014) isolated a pyrroloformamide, coded as vD-844 when it was first described (Von Daehne *et al.*, 1969), but escaped any sort of biological testing until then. With a purpose to fill this gap, this work uncovered an unusual function for the compound, characterizing the pyrroloformamide as a cytokinesis disrupting agent, thus leading to impaired mitosis and polyneucleated cells (Abreu *et al.*, 2014). Cell signaling alterations related to aberrant mitosis observed in cells exposed to pyrroloformamide are illustrated in Figure 5. Besides that, which is certainly the most stimulating finding of the investigation, it is worth mentioning, particularly in this context, the improvement in compound yield by using Amberlite XAD resin in the culture broth of the bacteria. This resin is capable of adsorbing organic compounds from aqueous solution, further concentrating the metabolites produced in the bacterial culture and enabling an efficient compound recovery.

A second round of bacterial isolation recovered 20 new strains, and 11 were followed up for their bioactivity profile. Five closely related *Micromonospora* sp. strains displayed a noteworthy *in vitro* cytotoxicity against tumor cells, while the extract obtained from *Micromonospora* sp. BRA006 (previously and therein codified as EVA0109) was the most potent (Jimenez *et al.*, 2013). A further chemical characterization of this extract was conducted, only to reveal the presence of four novel anthracyclinones, two of which own a different and rare arrangement of carbonyl carbons (Sousa *et al.*, 2012).

Anthracyclinones are the aglycones of anthracyclines, which, in turn, own an anthraquinone nuclei attached to glycosidic residues. These make up for a star-studded group of microbial compounds, which include the drug doxorubicin and few others, due to its prominence in anticancer chemotherapy (Laatsch & Fotso, 2008). Anthracyclines are known to exert toxicity through DNA damage mediated by topoisomerase II inhibition, and the presence of glycosides are crucial for this activity as these residues attach the compound to the DNA and hold the oxidative anthraquinone nuclei in its target (Binaschi *et al.*, 2001; Priebe, 2000). In fact, anthracyclines may be up to 100 times more potent than their aglycone counterparts (Dessypris *et al.*, 1988). The aglyclones isolated from the ascidian associated strain only showed moderate to low cytotoxicity (Sousa *et al.*, 2012), which does not sufficiently justify the strong activity displayed by the crude microbial extract. In light of this, one hypothesis to be considered is that the bacteria are indeed producing cytotoxic anthracyclines, however the glycosidic moieties of these molecules were lost during the extraction and isolation processes, thus yielding the aglycones anthracyclinones. This premise is being addressed and tested through a genomic framework. The main goal is to mine for gene clusters within the bacteria genome that carry information to support or disprove the biosynthesis of glycosylated anthraquinones.

**CONCLUDING REMARKS AND PERSPECTIVES**

Since the beginning of its exploration, marine biodiversity has proven to be a source of rich and unique chemical structures with several biotechnological applications (Centella *et al.*, 2017; Ferraro *et al.*, 2010). The same is true when we narrow our focus to ascidians, which have proven to be one of the most prolific invertebrates for the discovery of molecules with biomedical properties (Dou & Dong, 2019; Jimenez *et al.*, 2020). This review discussed merely a small part of the knowledge generated through the years in order to provide plausible explanations for ascidians’ valuable natural richness. Nevertheless, it further
reflects on the numerous genes and biochemical processes hosted by these animals, which evolved through billions of years, backing them as precious sources of information and innovation. In addition, the yet limited exploration of this resource, particularly in Brazil, reveals that the economic potential housed in the country’s megadiversity, crosses the Amazon and Atlantic rainforests and reaches the coastline.

New approaches for investigation and sustainable exploration of marine biological resources will certainly address new insights in this research field, while also encouraging biodiscoveries. In this sense, it is important to emphasize that the intrinsic biotechnological value of marine holobionts, including ascidians, should be extended to their microbiota. Microbes represent an ecologically sustainable source and, moreover, a supply platform for bioactive natural products, as it reduces the impacts of extractivism to a minimum and consents to amounting biological material biomass as needed without continuously resorting to the natural environment. Besides an ecological advantage, there are circumstances where commercial supply of microbial compounds can be guaranteed directly from their respective producer. That is the case with staurosporine, a natural molecule found within the extracts of the ascidian *E. vannamei* and associated bacteria *Streptomyces* sp.

Staurosporine is not a pharmaceutical product and, therefore, does not require a production pipeline for bulk quantities, however, is widely used as a scientific tool in experimental assessments in areas such as cell biology and pharmacology. Sigma-Aldrich, represented by MilliporeSigma, as informed in their website (Sigma-Aldrich, 2021), sells 1 mg of analytical grade staurosporine for U$ 1,170.00. More importantly, most natural products that serve as scientific tools or other sorts of applications can be high-valued products, worth, literally, many thousand times their weight in gold. Therefore, an eco-sustainable systematic continuous marine bioprospective enterprise could indeed support a profitable trade. Once a functional molecule, novel or known, is associated with a Brazilian microbial producer, it could be streamlined into production then marketed, endorsing at least two advantages: reducing costs of science projects in the country and funding conservation efforts for the region and peoples that provided and care for the biological material.

In this scenario, omics tools, and even multi-omics approaches, have been making their way into the study of natural products, unveiling a myriad of biosynthetic genetic clusters (BGCs) and putative metabolites yet to be discovered. The analysis of complex systems, as holobionts, using omics tools, are given a much more accurate idea of the hidden genetic diversity inside one single invertebrate species (Chen et al., 2018; Tianero et al., 2015). Besides, there are an increasing number of techniques aiming at heterologous expression of cryptic or silent BGCs (Kang & Kim, 2021; Yamanaka et al., 2014), making the exploration of these holobionts systems a boundless enterprise. These frameworks are also being tried as means to rationalize biodiscoveries by prioritizing samples and avoiding redundancy through metabolite profiling and annotation, aiming at progressing at a faster pace towards that envisioned outcome (Wolfender et al., 2019). These strategies, based mostly on genomic and metabolomic analyses, generate large amounts of data on the investigated samples which, assisted by bioinformatic gears, can subsidize further evidence on other important matters, such as detecting early responses of the marine community to climate changes, pollution and environmental degradation, thus enabling mitigation and damage minimizing measures to be immediately launched.
In light of this, we can conclude that bioprospection and biotechnological investigations are of great importance for breeding another layer of knowledge and worth about marine organisms through questions not typically asked in biology or ecology-based frameworks. Furthermore, these biodiscoveries, and the potential thereof of organisms and environments, may further serve as means to add value, awareness and even funds for marine conservation enterprises, thus nearing achievement of goal 14 of the United Nations Sustainable Development Goals.

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