McNair Research Journal SJSU

Volume 13 *Spring 2017* Article 4

2017

HIV-1 Protease Inhibitors From Marine Brown Alga: A Literature Review

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Cover Page Footnote
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Chemical Engineering

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Biography

Eric Nunez Aguilar is a junior at San José State University studying Chemical Engineering with an emphasis in biochemical engineering processes. Eric comes from a large Mexican American family he will be the first in his family to graduate from a four-year institution. Additionally, Eric hopes to be the first in his family to obtain an MD-PhD at a graduate school. Eric has a passion for research and has conducted biochemical and biochemical engineering research for more than three years. Eric's research interests are infectious diseases, protein engineering, bioinformatics, and marine pharmacology. Eric is particularly interested in the discovery and development of bioactive chemicals that can inhibit infectious diseases from maturation and potentially provide cures.

HIV-1 Protease Inhibitors From Marine Brown Alga: A Literature Review

Abstract

The human immunodeficiency virus type 1 (HIV-1) causes an infectious disease that if left untreated can progress to acquired immunodeficiency syndrome (AIDS) and be fatal. Finding a cure and more treatments for HIV has become a top priority in medical research, and due to the cost of synthetic HIV medication, finding a low-cost alternative is essential. Marine pharmacology has provided a possible solution to costly HIV medication through compounds derived from marine brown algae that inhibit the HIV-1 protease (PR). The objective of this study is to emphasize the necessity for further research in HIV-1 protease inhibition using marine-wildlife-derived compounds. In order to better understand the process of protease inhibitors, I will investigate the process of producing and purifying HIV-1 PR, extracting and isolating brown algal compounds, and the assays used to test the inhibition effects of the brown alga compounds. This study demonstrates the potential of marine pharmacology as an inexpensive alternative to synthetic pharmaceuticals for HIV-1 PR inhibition.

I. Introduction:

The HIV-1 is a viral infection that can be transmitted through direct exposure to infected bodily fluids. Global HIV and AIDS statistics indicate HIV has infected about 38.1 million people, with 36.9 million people and 2.6 million children seeking treatment. If HIV is left untreated, the virus can cause acquired immune deficiency syndrome (AIDS), which has caused about 25.8 million deaths due to AIDS-related illnesses. [6] HIV/AIDS is an illness people must live with for the rest of their lives. However, the medication to keep people healthy is quite expensive. [18] This makes a cure for HIV desirable, and researchers have been struggling to find a quick solution to prevent this virus from full maturation.

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DOI: 10.31979/mrj.2017.1304

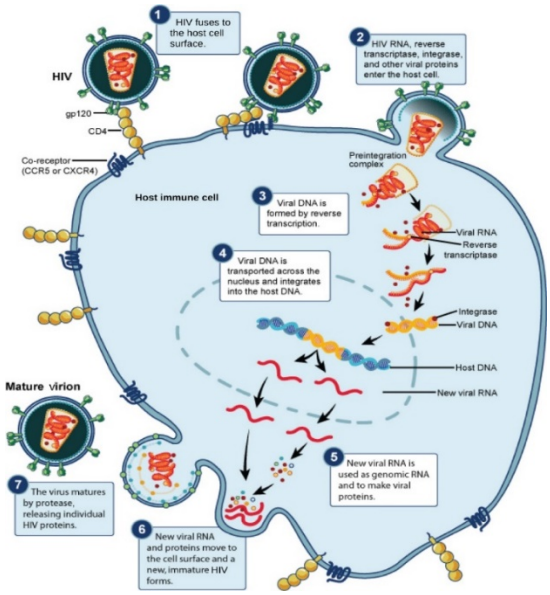

Figure 1: This diagram depicts the process in which a healthy CD4 T Cell is infected by HIV-1 and then used as a viral host to produce mature HIV-1 virions.
(IMG: https://cooljargon.com/ebooks/biology/m44599/index.cnxml.html)

The process in which an HIV-1 virion infects its host, the CD4 T Cell, is through seven steps depicted by **Figure 1**. First, the virion must fuse to the cell membrane of the CD4 cell through the CD4 binding site—illustrated as 4 yellow circles on the CD4 cell—where the co-receptor will help fuse the viral membrane to the CD4 cell membrane. Once attached to the membrane, the HIV proteases (PR) within the virion cleaves the Gag and Gag-Pro-Pol polyproteins into the following matrix (MA), capsid (CA), nucleocapsid (NC), and P6. The PR is also responsible for cleaving enzymes such as more proteases (PR), reverse transcriptase (RT), reverse transcriptase-RNase (RT-RH), and integrase (IN). The proper cleavage of these polyproteins is essential for the maturation of a virion. [10][9] Then, the PRs break away the capsid to expose the viral RNA, where reverse transcriptase will construct viral DNA, which integrase will insert into the host DNA. This results in a mutation in a CD4 cell that helps replicate viral proteins and RNA. This new viral RNA is transported to the walls of the CD4 cell by viral proteins in order to start the production of new, mature

HIV-1 virions that are released after maturation. This is where the CD4 host cell dies due to the crowding of the cell with virions, causing the cell to rupture and release large amounts of mature virions.

This process has allowed researchers to create preventative treatment and lifelong treatment for those already infected. The understanding of the mechanisms of HIV-1 infection has contributed to research in order to one day create a vaccine for HIV.[25][4] In **Figure 2**, the current inhibitors in treatment are indicated in red text, along with additional vocabulary necessary for understanding the structure of the HIV-1 virion. The target for most medication for HIV-1 is PR inhibitors, since inhibiting the protease will prevent virions from reaching maturation before infecting the host cell. Other inhibitors can target the entry of the virion by inhibiting the virus-cell membrane fusion. [23]

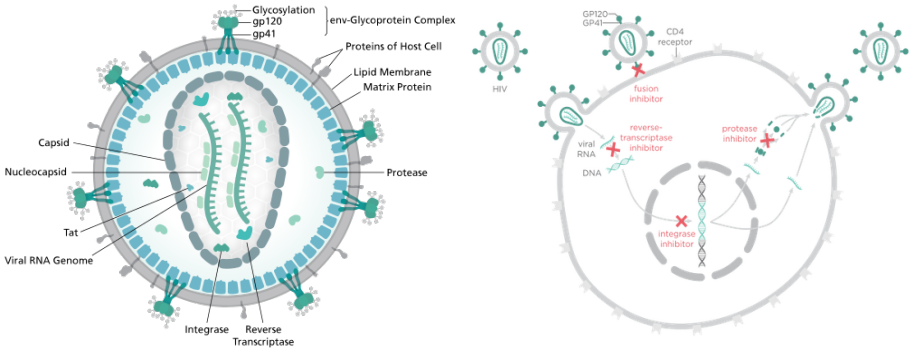

Figure 2: HIV-1 virion diagram depicting all essential components for infection of CD4 T Cell host (left). The inhibition of these components is essential in creating treatment and potentially a cure for HIV-1 (right).
(IMG: http://www.forbes.com/sites/quora/2016/09/29/these-two-promising-treatments-could-change-the-fight-against-hiv/#5f2ca215537b).

In this review, I will investigate the natural marine products used in HIV-1 PR inhibition in order to review the current understanding of how natural products are used in pharmaceuticals. I will look into the host system selection process, the production of PR, the isolation and characterization of PR from host cells, the extraction of bioactive chemicals from marine

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DOI: 10.31979/mrj.2017.1304

plants, and the testing of the marine plant natural products HIV-PR inhibiting effects. This article will illuminate the interdisciplinary cooperation between biochemical engineering and natural product research to help provide a better understanding of current biotechnology for large-scale viral protease production and strengthen the knowledge known about marine-plant-derived pharmaceuticals.

II. HIV-1 PR production from bacterial vectors

The production of the PR is a long and tedious process that can sometimes yield little to no PR if using the wrong host cell body or other conditions are not met. The organisms available for protein synthesis that are readily available consist of bacteria, yeast, filamentous fungi, and unicellular. [21] However, for this particular peptidase, the E. coli host system has been well documented and continues to show results in the literature. This section will review the production of viral protease from literature and look into the process of producing PR.

Production of PR from E. coli is a reoccurring theme in most literature on producing HIV-1 PR in recent years, and in this process the type of PR is selected based on the compatibility of the strain of E. coli to optimize the yield of HIV-1 PR. [5] For example, Nguyen et al. used a gene sequence cloned from a Vietnamese HIV-1-infected patient to obtain a native HIV-1 protease gene. The HIV-1 PR gene then had to be amplified and ligated using polymerase chain reaction (PCR). This process makes sure the gene of interest is amplified and isolated. In this scenario, the PR gene is located in between the Gag and Pro gene. The PCR process removes Gag and Pro genes and amplifies the desired gene by using digestive enzymes that cleave these unwanted genes. The gene had to be modified to make purification much simpler by adding a 6X His tag to the C-terminus and had to be encoded with an auto cleavage site at the N-terminus using designed primers. Then, it was cloned into a pET32a (+) plasmid containing the HIV-1 protease-encoding sequence along with sequences encoding an auto cleavage site GTVSFNF at the N-terminus. The plasmid also contained a TEV plus 6× His tag at the C-terminus and was expressed using Escherichia coli BL21 DE3 strain as the host cell for the expression of this PR. Nguyen had 9 plasmids from which only the highest expressed plasmid was selected

for the *E. coli* transformation. Through this process, Nguyen et al. expressed about 4 mg of protein per liter of cell culture. This article provides strong evidence that production of the HIV-1 PR is expressed and cultured in the *E. coli* bacterial host system.

The process detailed by Nguyen et al. shows the possibility of obtaining this PR using *E. coli*, and this creates room for optimization of this process. Bashiri et al. indicates that the *E. coli* BL21 DE3 strain is problematic due to the leakage of expression in lac promoter and T7-based systems. He suggests that the BL21-AI strain might lead to tighter regulation in expression and using a rhaBAD promoter to increase protein accumulation, protein solubility, and cell fitness. As for affinity tagging, the 6XHis tag used in Nguyen's experiment can be replaced with fusion partners, which consist of fusion proteins that reduce cost in purification of PR and increase the yield of PR produced. [3][21]

III. Purification and characterization of HIV-1 PR

This section will cover the purification process and will investigate current techniques used for characterization of the HIV-1 viral protease. Affinity column chromatography is a process in which proteins—which are removed from their host cells using lysis buffer to break the cell walls—are isolated from undesired compounds and contaminant proteins using high affinity resin beads in a column. This process consists of pumps that contain an elution and binding buffer, resin beads that bind to the 6XHis tag on the desired protein, and collection tubes to collect the desired protein. These components can all be found in a Fast Protein Liquid Chromatography (FPLC) instrument which monitors the compounds in the lysed and suspended cell pellet using a UV detector or specified light detector that can detect when a protein of a given or desired wavelength is being eluted from the column. Maseko and Nguyen both used column chromatography for purification and sodium dodecyl sulfate polyacrylamide gel electrophoresis for characterization of their proteases. This section will highlight both processes, along with current techniques that can strengthen their results.

Purifying PR using affinity column chromatography is a common technique used in the isolation of proteins from contaminants. Maseko et al. used a Q

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DOI: 10.31979/mrj.2017.1304

Sepharose Fast Flow anion exchange (QFF) and GSTrap column chromatography. QFF columns bind to charged biomolecules, in this case, the HIV-1 PR and other charged proteins. This is why there is another column GSTrap that binds specifically to the glutathione S-Transferase (GST) tagged proteins. [14] Maseko used a different plasmid with his GST tag based on his HIV-1 PR. The last affinity column selectively isolated only proteins with the tag, which yields a higher purity in his final product. Nguyen et al. used a 2 Tandem QFF similar to what Maseko used in order to isolate all charged biomolecules. However, Nguyen then used a Ni-Sepharose column in order to isolate her HIV-1 PR with a 6XHis tag.[16] The purification step of this PR demonstrates the importance of purification tags and purity in the final product. The two column steps help reduce the contamination in the PR final samples and help in concentrating the final product.

Characterization of PR using SDS PAGE and mass spectrometry is a common process for quickly analyzing the PR, the quickest being SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE is a common procedure, where protein samples are characterized based on the size of the protein. The accuracy of this process can be intensified if the size of the protein is known. Maseko et al. detail the PR sizes for his experiment by comparing his two PR variants with his control wildtype PR, which are 11.5, 11.6 and 11.7 kDa. [14] However, Nguyen et al. was only interested in the single band of approximately 13 kDa since she was not testing inhibition effects on other HIV-1 PR variants.[16] The most accurate method to characterize a protein is through mass spectrometry, which is the detection of mass isotopes of a given molecule. The purity of the sample and control is paramount to getting the best results. The process of obtaining a mass spectra of a sample is long and tedious, consisting of method development and experiment runs that can take hours to process. Loo et al. [12] demonstrate a mass spectra of the HIV-1 PR used in the experiment, where they detail the procedure of how they obtained their data (**Figure 3)**. This procedure is essential in priming the mass spectrometer and prepping the PR samples.

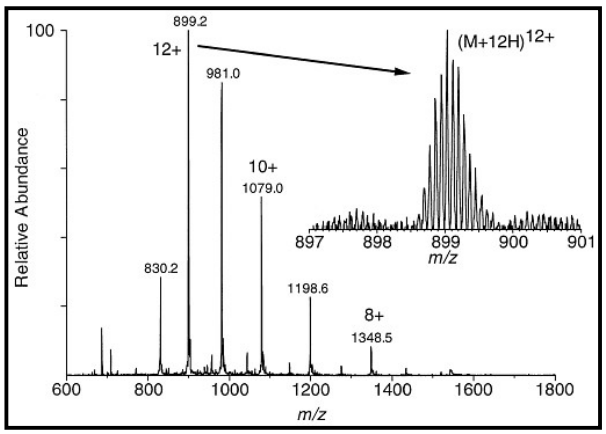

Figure 3: This image depicts the HIV-1 PR mass spectra, where you can note the isotropic resolution of this M+12H ion. [12]

IV. Marine plant natural product extraction from marine wild life with potential anti-HIV properties

In the discovery of new pharmaceutical or nutraceutical compounds, these compounds must be either synthesized in a laboratory or extracted from the natural source. This section will look into the extraction of natural compounds from marine wildlife, and in particular, plants and algae that have pharmaceutical applications such as; antioxidants, anti-inflammatory, anti-viral/retroviral, and anti-fungal.[15][22] This scale of extraction for pharmaceutical purposes would need to be conducted on a much larger scale if it were to be used for drug discovery research. Malve speaks of marine pharmacology as a viable source for alternative drug discovery for new illnesses emerging from marine microbes, which in this section pertains to marine alga. [13][7] Uzaira et al. indicate that microorganisms like alga have exhibited in-vitro inhibition of HIV-1 replication cell lines. [27][19] It is through marine organisms that most of the answers to infectious diseases can be found. However, there is still more research needed in this field to identify and isolate these compounds; **Figure 4** illustrates the process and ways that these compounds are characterized.

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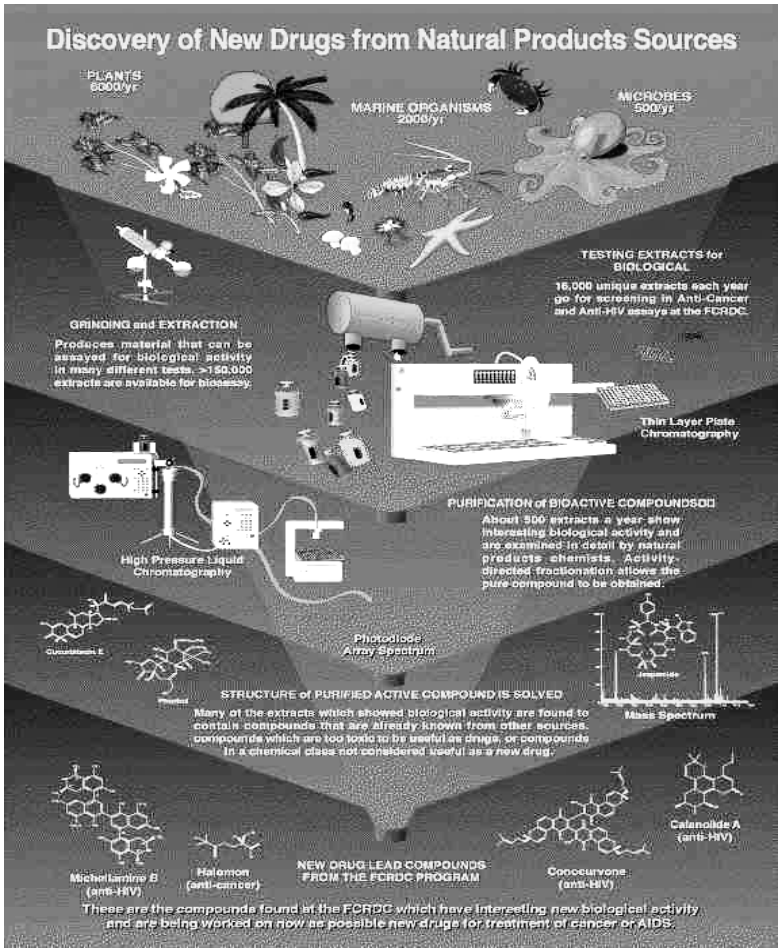

Figure 4: This picture illustrates the process of extracting, isolating and testing marine pharmaceuticals using characterization instrumentation like high performance liquid chromatography and mass spectrometry.
(IMG: https://npsg.ncifcrf.gov/DISCOVER.JPG)

Potential viral protease inhibitors are found in algal natural products in the pacific coast of Japan and South Korea called *Ecklonia cava*. This brown, edible algae consists of phlorotannins; some are Eckol, 6, 6'-Dieckol, 8, 4"-Dieckol and 8, 8'- Bieckol. In particular, 8,8'-Dieckol is known to inhibit the protease more than all of the other derivatives because the inhibiting

effects on PR are greater than the other 3 phlorotannins, PR-IC50 81.5±9.6 μM.1. This indicates that this algal derivative should be further investigated to determine whether it can be used for PR inhibition. Artan et al. mentions that 8, 8'-bieckol has a much higher inhibiting effect. [2] In this experiment, the extraction and isolation of 8, 8'-bieckol from Ecklonia cava is run by washing *E. cava* with water three times followed by drying 1 kg of the washed sample at 60℃ for 12 hours. Following that, the dried sample is ground and then extracted with methanol (3x). This methanol extract is then dissolved in water and partitioned with n-hexane, which results in an aqueous layer that is partitioned with ethyl acetate. The organic solvent fraction is concentrated in vacuo and divided into ten fractions on a silica gel column chromatography (Merck, 230—400 mesh, 300 g) using trichloromethane-ethyl acetate-methanol mixtures of 50 : 2 : 1 (f. 1), 25 : 5 : 1 (f. 2, f. 3), 10 : 5 : 1 (f. 4—f. 6), 5 : 5 : 1 (f. 7, f. 8), 100% MeOH (f. 9, f. 10); 1 l each.[2] The desired compound is obtained from fraction 5 through recrystallization in methanol/water. The information regarding the method for extracting this compound is essential in order to purify the compound out of *E. cava*. This compound can also be bought from 3B Scientific Corporation (3BSC SKU# PL074128).

V. HIV-1/2 PR inhibition analysis

Finding the right assay to quantify the results in inhibitor experiments can be tricky due to the desired conditions of the PR and marine natural product optimal conditions differing. Furthermore, determining the type and how much the natural product is inhibiting takes time, and sometimes the current method used is not the most accurate. This is where technology is involved and can determine whether an experiment is reliable based on the instrument used. In this section I will go over two assays: one that used high performance liquid chromatography (HPLC) to determine the inhibition effects and the fluorescence resonance energy transfer (FRET) assay that is commercially used in the pharmaceutical industry. FRET is measuring the amount of a particular compound given a specific wavelength that the compound can be detected in.

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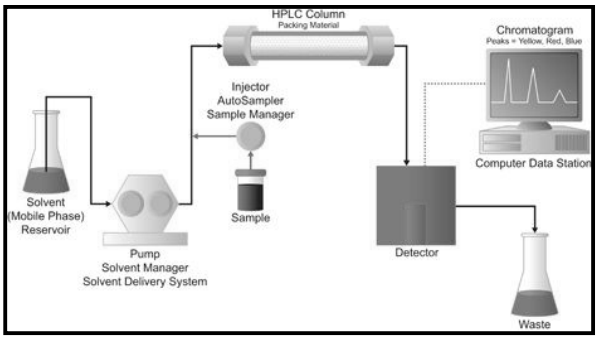

Figure 5: Process flow diagram of an HPLC and the components it has. (IMG: http://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055&locale=en_US)

The HIV-1 protease assay using HPLC: HPLC is an instrument used to measure the amount of compounds within a solution using a column that separates each compound; these compounds go through a specific wavelength, where they can be detected and quantified (shown in **Figure 5**). Ahn et al. used HPLC to determine the amount of hydrolysate and remaining substrate from her inhibitor reaction, where the HIV-1 PR cleaves this substrate and the products are hydrolysate. The following conditions were set for this experiment: column, Inertsil ODS-3 (4.63150 mm, GL Sciences Inc., Japan); elution, a linear gradient of CH3CN (15→40%) in 0.1% TFA; injection volume, 20 ml; flow rate, 1.0 ml/min; and detection, 280 nm. The retention time for hydrolysate and substrate to be eluted is at 8.61 and 10.84 min, respectively. To determine the inhibition activity of the compound in PR, reaction was calculated using this equation: % inhibition = 100X (Acontrol-Asample)/ (Acontrol), where A is a relative peak area of the hydrolysate; Acetyl pepstatin (Bachem AG) was used as a positive control in this assay. [1]

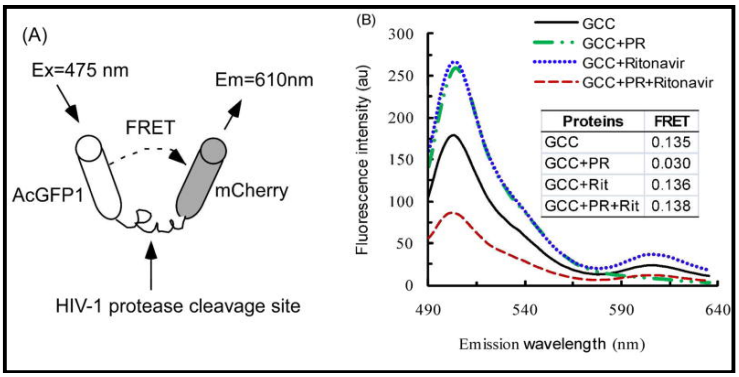

Figure 6: This diagram illustrates how the FRET assay works and the proper way of running controls and experimental trials. (A) This substrate fluoresces at an excitation of 475nm and an emission at 610nm, where if there is activity the substrate will not fluoresce. (B) The substrate (GCC) was tested by itself, substrate + protease, substrate + inhibitor (Ritonavir) and substrate + inhibitor + protease. [8]

The HIV-1 PR activity can also be analyzed using the fluorescence resonance energy transfer (FRET) assay specifically meant for testing HIV-1 PR inhibition compounds. The FRET HIV-1 PR assay works by using a substrate that fluoresces at a given excitation and emission wavelength. HIV-1 PR is determined to be active if there is no fluorescence, since the whole substrate must be intact to fluoresce. This FRET assay is used in industries to screen several HIV-1 PR inhibitors and is commercialized, making this a readily available option when screening for HIV-1 PR inhibitors.[8][29] This method is more preferable than HPLC since running HPLC can be costly and takes more time to run effectively. The accuracy of a HPLC is what makes the HPLC method desirable; with FRET, the accuracy is more of a quick way to screen through several inhibitors. This is why using both would be beneficial by using FRET assay to screen through several marine natural PR inhibitors and then run a HPLC assay for the inhibitors that made it past the screening.

VI. Discussion

The literature in this review contributes a foundation to marine pharmacology and HIV-1 research. Nguyen's contribution to HIV-1 PR expression and production allows for reproducing the same method for

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future experiments like HIV-1 PR inhibitor simulations that are crucial in understanding the structural change of a PR when present with an inhibitor and catalytic response after HIV-1 RNA direct interaction.[19][17][20] Maseko and Nguyen both provide sufficient information to optimize current HIV-1 PR purification processes, suggesting new forms of purification like fusion protein tags, using different plasmids with better binding affinity for column chromatography.[24] Artan provides an extraction process that can be used to extract other algal natural products that have been used by other marine pharmacology researchers.[26][28] Additionally, HIV-1 PR assays have been created by researchers like Ahn's HPLC assay and Zhu's FRET assay; this creates a platform for testing inhibitors at a small to large scale. These contributions are what establish potential drug components in HIV-1 PR medication. These contributions to marine pharmacology and HIV-1 research are just the building blocks for new and improved processes for nutraceuticals.

The strengths and weaknesses of the literature can be addressed through further research in each process. The expression and production of HIV-1 PR can always be optimized through further investigation in plasmids with better binding affinity tags and designing primers that can amplify the PR gene more efficiently. The selection of better *E. coli* strains through large scale screening of different strains that do not have the leakage problems like T-7 and lac promoter strains. This can be addressed by screening through strains with a rhaBAD promoter to increase protein accumulation, protein solubility, and cell fitness. Purification and characterization of HIV-1 PR can be optimized by replacing affinity tags with fusion protein that can significantly reduce cost, and using SDS-PAGE and mass spectrometry together to characterize PR will only strengthen the literature. Extraction and isolation of marine plant natural products can be optimized by using industry-grade plant extracting units to provide for better purity and additional controls for conditions specific to each compound. The HIV-1 PR assay development has been optimized through FRET and HPLC methods that detail the processes and controls using reliable instrumentation that are standard in research. The next steps in HIV-1 PR protease inhibitor research would be to further investigate other marine organisms and test their inhibitor activity of HIV-1 PR.

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DOI: 10.31979/mrj.2017.1304