RAPID ASSAYS TO SCREEN MARINE FLORA AND FAUNA FOR THEIR INHIBITORY ACTIVITY AGAINST ANTIMICROBIAL ENZYMES

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Abstract

The worlds land and sea represent a limitless reserve of natural, biological and potentially active useful pharmaceutical products. Renewed interest in drugs from natural reserves, advances in chemical separation and analysis have identified hundreds of unique exotic physiologically active substances with antimicrobial, anti-tumor and other potentialities more rapidly, from terrestrial plants, marine flora and various classes of marine fauna. Development of practical and rapid methods for detection of plant products with activity against infectious disease producing organisms is the need of the hour. Rapid assays for antiviral studies using isotopic reverse transcriptase, E. coli DNA polymerase and HBV DNA polymerase inhibition assays were standardized. Extracts of marine flora and fauna collected under the DOD National project were assessed. Of the extracts assessed for virus specific enzyme inhibitions, 24.35% possessed RT inhibition activity, 15.83% E. coli DNA polymerase Inhibition activity, 42.72% of 35 extracts tested possessed HBV DNA polymerase inhibition activity and 11.42% of 35 extracts tested possessed inhibition activity against all three enzymes tested. The great potential of ocean's flora and fauna is there to be used for its antimicrobial activity in general and antiviral activity in particular, which will suit our ever increasing need, is to be harnessed for the welfare of the human community at large.

Key words: HBV and *E. coli* DNA polymerase inhibition assay, Rapid antimicrobial assay, RT inhibition assay

INTRODUCTION

Relationship between man and plants has been very close throughout the development of almost all civilizations. Every tribe or race has had its own way of curing afflictions of disease which depends very much on the practice, belief and knowledge they possess. Besides chants, prayers and other practices, humans utilize various resources, especially flora and fauna around them to a greater extent to achieve this. The worlds land and sea represent a limitless reserve of natural, biological and potentially active useful pharmaceutical products. Renewed interest in drugs from natural reserves, advances in chemical separation and analysis have identified hundreds of unique exotic physiologically active substances with antimicrobial, anti-tumor and other potentialities more rapidly, from terrestrial plants, marine flora and various classes of marine fauna.

Preclinical studies on Spirulina, a unicellular cyanobacterium has shown to have several therapeutic attributes like cholesterol regulating, antiviral and anti-mutagenic properties[1]. Aqueous extracts of *Haslea ostreania* and *Polysiphonia denulata* from the Black Sea coast have shown to inhibit HSV in cell culture by demonstrating reduction in cytopathic effect caused by the virus [2, 3].

Inhibitory activity of marine algae were investigated and it was found that cyanovirin-N, a 11 kDa protein, from blue green alga irreversibly inactivated HIV and also aborted cell-to-cell fusion and transmission of HIV, due to its high affinity interaction with gp120 (4). Other cases of antiviral activity against HIV, HSV and other viral adsorption processes by marine flora and fauna have been reported [4, 5, 6].

Development of rapid methods for detection of infectious disease producing agents is the need, and trend of the hour, for early diagnosis. Many such methods have found success in recent times. In the present study we outline our experience in standardization and evaluation of few such methods, viz., ATP reduction assay for antibacterial testing, and reverse transcriptase, E. coli and HBV DNA polymerase inhibition assays for antiviral testing, that have been adapted to evaluate the antimicrobial potentials of natural products in general and marine flora and fauna in particular.

MATERIALS AND METHODS:

The work on standardization and evaluation of rapid assays for marine flora and fauna was carried out in the Microbiology Department, Dr.ALPGIBMS, University of Madras, Chennai -600 113. Extracts of marine flora and fauna collected under the Department of Ocean Development National project were assessed. A cohort of samples showing positive and negative HBsAg binding activity was picked randomly and was included in this study.

Isotopic Enzyme Assays

A total of 221 extracts from marine flora and fauna were tested. Three different isotopic assays were standardized to measure reverse transcriptase (RT), *E. coli* DNA polymerase and HBV DNA polymerase inhibition properties of the extracts from the marine organisms. The enzymes assessed for the study are most required for the replication of the respective microbial agents.

Standard Isotopic RT-Inhibition assay:

It was carried out as described by Ono *et al*[7]. The assay was carried out establish the reverse transcriptase enzyme inhibitory activity of the marine extracts. The assay was performed in a reaction mixture that contained the following in a final volume of 50 µl. Tris HCl 50 mm, dTTP 1.5 mM, Poly(rA)$(dT)_{12\text{-}18}$ 10 µg/ml, BSA 10 µg/ml, ^{3}H-TTP 0.5 mM, KC1 75 mM, DTT10 mM, $MgCl_2$ 3 mM and MMLV-RT 1 Unit.

In the test, a known concentration of extract was added to the reaction mixture and incubated. Similarly a positive control (0.1 µg/ml AZT), a negative control (distilled water) and a solvent control (solvent used to extract) were set up. Each set of test and controls were run in triplicate. After 30 minutes the reaction was stopped by adding 10µl of ice cold EDTA (0.2 M) and immersing the mixture in ice immediately and simultaneously processed for radioactivity measurement.

***E. coli* DNA polymerase inhibition assay**:

The procedure followed was as described by Ono *et al*[7]. The assay was carried out establish the *E .coli* DNA polymerase enzyme inhibitory activity of the marine extracts It was performed in a reaction mixture containing the following in a final volume of 50 µl: Tris HCI (pH 7.5) 50 mM, DTT 5 mM, Tritiated TTP 10 pM, dCTP, dATP, dGTP 10 pM each, BSA 200 gg/ml, MgC12 5 mM, KCI 100 mM, Activated calf thymus (CT) DNA 2 pg/ml and *E coli* DNA polymerase-I 1 Unit. Activated CT DNA was prepared as described by Goulian and Heard[8].

After the addition of the enzyme, the reaction mixture was incubated at 37°C/30 minutes. This acted as the negative control. In the test a known concentration of the extract was added and incubated. Both the test and control reactions were stopped by adding 20 µl of 0.2 M EDTA, after which they were spotted onto a Whatman DE81 filter paper discs and processed for radioactivity measurement. Each batch of test and control were run in triplicates.

HBV DNA polymerase inhibition assay:

The assay was carried out establish the HBV DNA polymerase enzyme inhibitory activity of the marine extracts

Virus preparation: Pretritrated HBsAg and HBeAg positive serum was centrifuged at 35,000 rpm for 3 hrs using SW41 rotor. The pellet was washed in PBS and again centrifuged at 35,000 rpm. The pellet got in this was dissolved in PBS and stored at 20°C.

The procedure followed was as described by Lofgren *et al*[9]. Prior to the assay, the virus preparation was pretreated with 1/8 volume of 2% mercaptoethanol and 10% NP_{40} for 15-30 minutes at room temperature. Aliquots of 25 µl were incubated at 37°C/3 hours together with 25 µl reaction mixture containing Tris HC1 (pH 8.0) 100 mM, $MgCl_2$ 20 mM, KCl 200 mM, dNTPs 10 mM each and 3H dTTP 10 mM and 25µl of DNase and RNase free water, or a solution of the substance to be studied. After incubation 10 µl of 0.2 M EDTA was added and spotted onto a Whatman DE81 filter paper discs and processed for radioactivity measurement.

All the chemicals for the three assays were procured from Sigma Chemicals, St.Louis, USA**.**

Processing for radioactivity measurement:

After termination of the reaction in the above isotopic assays, DNA was precipitated using 10 µl of cold 5% TCA and 0.1 M sodium pyrophosphate. 50 µl of the reaction mixture was then filtered through Whatman DE51 filter paper. The filter paper was later washed thrice in 3 ml of 5% TCA and, three times in absolute alcohol. The filters were then air dried, and radioactivity measured using a toluene-based scintillation cocktail[7]. A reduction of 50% or more in the radioactive count in comparison to the control was taken as evidence of inhibitory activity.

RESULTS:

Isotopic Enzyme Assays

Of the 221 extracts tested for RT inhibition 24.89% showed the activity. 23.47% of flora and 26.02% of fauna tested were found to possess the activity with an MIC ranging between 100 - 1600µg/ml (Table 1).

221 extracts from marine flora and fauna were tested for *E. coli* DNA polymerase inhibition. Of them 15.83 % showed the activity with MIC ranging from 100 - 1600µg/ml (Table 2). The inhibitory activity was observed in 20.41% of flora and 12.20% of fauna tested.

Thirty five extracts that showed *E. coli* DNA polymerase activity were tested for their HBV DNA polymerase inhibitory potentials. Of them, 42.72% possessed the activity with MIC ranging from 250 - 1000µg/ml (Table 3).

On comparison of the enzyme inhibitory potentials of these 35 extracts, four of them possessed all the three, two of them possessed RT and E. coli –DNA polymerase inhibitory activity, 12 of them possessed *E. coli* and HBV DNA polymerase inhibitory activity and 17 of them possessed only *E. coli* DNA polymerase inhibitory potential (Table 4).

Table 1: Isotopic RT inhibition assay

	Flora (%) (n=98)	**Fauna (%) (n=123)**	**Total (%) (n=221)**
Isotopic RT inhibition	23 (23.47)	32 (26.02)	55 (24.89)
MIC range µg/ml	100 to 1600	100 to 1600	

Table 2: *E. coli* DNA polymerase inhibition of marine organisms

	Flora (%) (n=98)	**Fauna (%) (n=123)**	**Total (%) (n=221)**
***E. coli* DNA polymerase inhibition**	20 (20.41)	15 (12.20)	35 (15.83)
MIC range µg/ml	100 to 1200	100 to 1600	

Table 3: HBV-DNA polymerase inhibition of marine organisms

	Flora (%) (n=20)	Fauna (%) (n=15)	Total (%) (n=35)
HBV DNA polymerase inhibition	9 (45.00)	7 (46.60)	16 (42.72)
MIC range µg/ml	250 to 1000	500 to 1000	

Table 4: Comparison of HBsAg binding and DNA polymerase activity

RT, *E. coli* DNA polymerase & HBV DNA polymerase inhibition positive	RT, *E. coli* DNA polymerase inhibition positive	*E. coli* DNA polymerase & HBV DNA polymerase inhibition positive	Only *E. coli* DNA polymerase inhibition positive
4 (11.42%)	2 (5.71%)	12 (34.28%)	17 (48.57%)

DISCUSSION:

Natural product research is turning to marine flora and fauna as a source of natural products and is currently in preclinical and clinical evaluation. Numerous traditional medicinal plants have been reported to have strong antimicrobial activity and have been used to treat animals and humans suffering from infectious diseases. Rapidity in search for newer products from natural sources has reaped its benefits.

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat humans and animals[10, 11, 12, 13]. There are also many groups marine flora and fauna, including cyanobacteria, showing antiviral, anti HSV, anti HIV and anti influenza activity [4, 5, 6].

Inhibition of RT by potential antiviral substances has been analyzed by several methods. The isotopic RT inhibition assay uses tritiated thymidine triphosphate which is incorporated into the template during the reaction. The difference in the count after addition of the compound reveals the level of inhibition of RT (8). Colourimetric assays are similar to the isotopic method except that RT activity is measured by incorporation of biotin digoxigenin labeled dUTP which is estimated photometrically by immobilizing the labeled DNA after reverse transcription. This technique has been designed as an ELISA by Eberle and Seibl [14].

The isotopic RT inhibition assay is a highly sensitive and quantitative method. Ono *et al*[7] studied RT inhibition activity of various Asian and Japanese herbal extracts and reported that 19 out of 40 extracts tested were active at an MIC ranging from 100 to 500 µg/ml. Inhibition of RT by flavonoids were also studied by Ono *et al* (7) and Spedding *et al* (15). Higuchi *et al*[16] reported the RT inhibition activity of anthraquines on HIV RT and RAV2 RT and MIC ranged from 7.7 - 300 µg/ml. In our study the inhibition levels varied between 100-1600µg/ml[17], which correlates well with other studies. Much lower inhibition levels could have been achieved, had been all the extracts were prepared in our lab, which would have overcome the loss of activity that occurred during the transport and other related factors.

Hepatitis B has a major implication to both the developed and developing world because of millions of chronic virus carriers, besides symptomatic disease population. Despite the introduction of effective HBV vaccines, there is a need for effective therapy since the vaccines are of no use to symptomatic and asymptomatic HBV carriers. The lack of tissue culture systems for HBV propagation is a handicap in the search for anti-HBV agents with potential use against the different syndromes caused by HBV. The search for active compounds against HBV by target enzyme screening is considered as a preferred strategy. Since replication of hepadna viruses involves a viral DNA polymerase containing both DNA dependent and RNA dependent activity, this polymerase is a potential target for chemotherapy against hepatitis B. Thus investigators have used DNA polymerase inhibition techniques and RT-inhibition techniques as methodologies of evaluating potential anti-HBV compounds[9].

Of the 35 extracts which have shown *E. coli* DNA polymerase inhibition, only 16 possessed HBV-DNA polymerase inhibition property, some of them also possessing HBsAg binding property. While confirmation of these activities are still required either by animal models like

DHBV infected chronic ducks[18], WHBV infected chronic carrier wood chucks[11] and more recently HBV infected cell lines.

On confirmation of the drug potentials against HBV by the above models, they are to be subjected for toxicological studies, and before being made as candidate preparations for Phase I and Phase II clinical trials in human beings subject to ethical clearance.

In total the standard isotopic assays are highly sensitive and have been able to detect antiviral activity at very low concentrations. One should also take to consideration the labour-intensiveness and restrictions that are inevitable while using radioactive material. In addition the requirement of a liquid scintillation counter for estimating the amount of tritiated thymdine incorporation, an instrument that is quite expensive, should also be noted. Presently, the use of colourimetric assays has gained prominence, which is safe, and sensitive, to evaluate the enzyme based assays to study antiviral activities.

Advances in rapid assays and standardization of newer techniques have reached great heights as the advance in science is onto to a great leap. Newer rapid techniques that are reliable and efficient are now available. In addition, bioinformatics is playing a great role in quicker identification of the specific components/compounds with potential activity. Antimicrobial research is entering an era of the biochip technology, where the efficacy of a plant product against numerous organisms could be identified in a very short time period. In a few years to come identification of newer antimicrobials will overcome the draw backs of resistance to antimicrobials by the evolving pathogens, with great ease.

CONCLUSION

The rapid assays employed to assess the antimicrobial enzyme activities can be used as trust worthy methods to assess the antiviral properties of only marine flora and fauna, but for any plant material or pure compounds. It can be modified to use to non isotopic measurement protocols instead of the radioactive ones used in the study.

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