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STUDY OF ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF BIOACTIVE COMPOUNDS OF ISOLATED ACTINOMYCETES FROM DHOLAI BANDAR

M. A. TANDEL*, S. R. MAURYA AND R. G. DESAI

[1]Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management & Commerce, Valsad, Gujarat, 396001, India.

ABSTRACT

Actinomycetes are wide spread in nature and are virtually unlimited sources of novel bioactive compounds with many therapeutic applications. In the present study, we reported Antioxidant and Cytotoxic activities of bioactive compounds of isolated Actinomycetes. Total 6 marine Actinomycetes were isolated from five marine soil and five marine water sample of Dholai Bandar area of Valsad District, Gujarat, India. The isolates were tested with standard biochemical test and utilization of carbon sugar, to confirm the Actinomycetes. Metabolites Produced by using starch casein agar medium on shaker at 100 rpm within incubation periode 5-7 days at temperature 28˚C.The antioxidant activity of the isolates was showed that metabolites of ethyl acetate extracts have higher reducing power than metabolites of ethyl alcohol extracts. Among tested extracts, those of Actinomycetes strain W19 shows highest reducing power with absorbance of about 1.0. The invitro cytotoxicity assay on yeast *Saccharomyces cerevisiae* cell revealed that the secondary metabolites had the strongest cytotoxicity with Ic50 7.46µg/ml. The data from both assays showed that marine Actinomycetes can be used as the potential source of natural antioxidant and cytotoxic compounds.

KEY WORDS: ***Actinomycetes, Bioactive compounds, Reducing power, Cytotoxic activity, Antioxidant activity, Saccharomyces cerevisiae.***

M. A. TANDEL*

Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management & Commerce, Valsad, Gujarat, 396001, India.

***Corresponding Author**

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INTRODUCTION

Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Research into pharmacological properties of marine natural products has led to the discovery of many compounds considered worthy of clinical applications. There are great potential in bioprospecting from the sea and marine natural products research has just started to bloom. Today, marine sources have the highest probability of yielding natural products with unprecedented carbon skeletons and interesting biological activities. The marine environment supports a great biodiversity with a correspondingly great potential for the discovery of unique and pharmaceutically active secondary metabolites.[1] There are several organisms that can produce natural pharmaceutically active secondary metabolites, including Actinomycetes. Actinomycetes are Gram-positive, Filamentous eubacteria. They belong to Actinomycetaceae family. Some Actinomycetes are aerobic, anaerobic or facultative anaerobic. Some Actinomycetes species may form endospores. Actinomycetes are Ubiquitous occurring in soil, marine environment and in the human microbiota. Some species produce natural antibiotics, antioxidant, anticancer agents and other pharmaceuticlly useful compound. Selman Abraham Waksman (1940) with the help of Boyd Woodruff discovered the antibiotic actinomycin. Actinomycetes are useful source of novel secondary metabolites with a range of biological activities that may ultimately find application as Anti-infection, Antioxidant, Antimicrobial agent, Anticancer agent or other pharmaceutically useful compounds. Although heavily studied over the past three decades, Actinomycetes continue to prove themselves as reliable source of novel bioactive compounds. Recently, new targets have been added to the general screening like AIDS, immunosuppression, anti-inflammation, Alzheimer disease, ageing processes, some tropical diseases and resulted in discovery of several drugs.[2] The Genus of Actinomycetes, including *Streptomyces sp.* produce highest bioactive compounds and these bioactive compounds shows antimicrobial activity. Apart from antimicrobial activity they also exhibit antioxidant and cytotoxic activities. The Secondary metabolites are produce at stationary phase, and its production is increases by depletion of nutrient in their growth medium.[3] The *Streptomyces sp*. NBRC 13020 shows good amount of antimicrobial and antioxidant activities.[1] An Antioxidant is a molecule that inhibits the oxidation of other molecules. Antioxidant is mainly used for two different groups of substances: (1) Industrial Chemicals which are added to products to prevent oxidation. (2) Natural Chemicals found in foods. They are providing protection to humans against various infection and disease by inhibiting and scavenging free radicales. Example of Antioxidant produces by Actinomycetes: “Mylothiol”. Cytotoxicity defined as the possession of such desteructive action which lysis the cells or some antineoplastic agents that selectively kill cells. Cytotoxic agents produce by Actinomycetes are clinically useful antitumor drug. The high toxicity of this cytotoxic agent ususally associated with cancer chemotherepy drugs. Cytotoxic agent produce by Actinomycetes: Anthracyclines, peptides (bleomycin), ureolic acids, mitomycins and others. Various synthetic antioxidants have been used in stabilization of foods and synthetic chemotherapeutic drug in cancer treatment. But because of their toxic and more side effects the main objective of this work is the production and characterization of novel bioactive compound from Actinomycetes and screen their antioxidant and cytotoxic activities.

MATERIALS AND METHODS

Sample collection

Ten Marine soil and water samples (Five water and Five soil samples) were collected from Dholai Bandar area of Valsad District, Gujarat, India. The soil samples were collected in sterile plastic bag from depth of 15cm and the water samples were collected in sterile bottles. The samples kept in refrigerator for overnight and next day brought to the laboratory.[4]

Physiochemical Property of Samples

The Physiochemical property of water samples including pH, Temperature, TDS, TSS and Hardness were checked and pH, Temperature, colour for soil samples were checked.[5]

Isolation of Actinomycetes

Serial dilution of soil and water samples was done (10^{-1} to 10^{-6}) and 0.1 ml aliquots of each dilution was poured and plated on Starch casein agar plates (SCA). The plates were incubated at 28˚C for 7-15 days. The colonies were subcultured and maintain on SCA slants.[6]

Identification and characterization of the isolated Actinomycetes

The morphology of Actinomycetes were studiedalong with microscopic characteristics. The isolated Actinomycetes were biochemically confirmed and characterized.[7]

Production of secondary metabolites

Isolates were inoculated in 100 ml Erlenmeyer flask containing 50 ml of Starch casein broth (SCB). And incubated each flask at 28˚C on shaker at 100 rpm for 5 days. These inoculums were transferred to 250 ml of fermentation medium and incubated for 5-7 days at 28˚C on shaker at 100 rpm.[8] Finally, the culture broth was harvested by centrifuged at 12,000 rpm, 4˚C for 20 min and extraction of the secondary metabolites was carried out using ethyl acetate and ethyl alcohol as solvent as described in the next step.[3]

Extraction of metabolites

The supernatant was collected from broth and it was subjected to extraction of metabolites. From centrifuged culture broth supernatant was subjected to solvent extraction using ethyl acetate and ethyl alcohol solvent. Equal volume (1:1) of supernatant and ethyl acetate and ethyl alcohol solvent were taken in a separation funnel and agitated for about 30 minutes. The solvent layer was separated and the supernatant was again extracted with ethyl acetate and ethyl alcohol solvent. The solvent layers were pooled and evaporated to dryness at 40˚C.[3] The crude solvent extract thus obtained was screened for antioxidant and cytotoxic activities.[9]

Antioxidant Activity

The antioxidant activity was determined by Reducing Power method. The determination of Reducing Power activity was carried out as described by Oyaizu (1986).[10] In this method 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) were added to 1.0 ml of sample dissolved in distilled water. The resulting mixture was incubated at 50˚C for 20 min, followed by the addition of 2.5 ml of Trichloro acetic acid (TCA) (10% w/v). The mixture is centrifuged at 3000 rpm for 10 min to collect the 2.5 ml upper layer of the solution, mixed with equal volume of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v). The absorbance was then measured at 700 nm against blank.

Cytotoxic Activity

The cytotoxicity was determined according to the method with some changes.[11] Yeast *Saccharomyces cerevisiae* cell lines were grown in Glucose yeast extract agar medium (GYE). For cytotoxicity assays, cell were seeded into 96-well plates at a density of 1×10^8 cells/well and the ethyl acetate extract dissolve in distilled water in the volume of 10µl, 20µl, 30µl, 40µl and 50µl were added. The samples also included a 'blank' (medium and water) and 'control' (DMSO). After incubation period (2h at 45˚C) 10µl $K_3Fe(CN)_6$ (1% w/v) were added. The resulting mixture was incubated at 50˚C for 20 min, followed by the addition of 10µl Trichloro acetic acid (TCA) (10% w/v). The mixture was incubated at room temperature for 30 min. After that distilled water 10µl and $FeCl_3$ (10% w/v) 2µl were added. The absorbance was then measured at 570 nm using microtiter plate reader. The % cell inhibition was determined using following formula.[8]

$$\% \text{ Cell inhibition} = \text{Mean Absorbance of sample/Mean Absorbance of control} \times 100$$

RESULT

Isolation of Actinomycetes

A total of 10 marine samples have been collected from the different sites of Dholai Bandar of Valsad District, Gujarat, India for isolation of Actinomycetes. Physiochemical analysis were done of all collected samples before procedding further, Result shows in given Table 1 and Table 2. Prelimenary total 6 isolates of different Actinomycetes were isolated by using starch casein agar (SCA) supplemented with sea water. The colonial characteristics of isolated Actinomycetes were noted in Table 3. All the isolates were further studies by using Gram staining method to determined either it was Gram positive or Gram negative. By Gram staining, all the isolates were identified as Gram- positive, filamentous Actinobacteria.

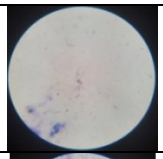
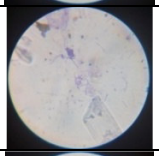
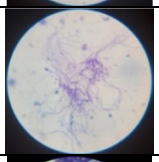
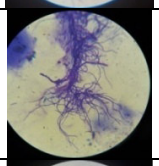
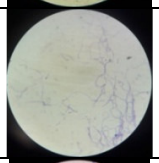
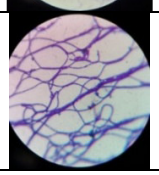
Table 1
Physiochemical analysis of water samples of Dholai Bandar area.

Sample No.	pH	Temperature	TSS	TDS	Hardness
1	6.5	33˚C	36666mg/lit	468mg/lit	3400mg/lit
2	6.5	31˚C	1000mg/lit	469mg/lit	2900mg/lit
3	6.5	33˚C	30933mg/lit	405mg/lit	3360mg/lit
4	6.5	31˚C	1000mg/lit	460mg/lit	3700mg/lit
5	6.4	32˚C	1300mg/lit	398mg/lit	3180mg/lit

Table 2
Physiochemical analysis of soil samples of Dholai Bandar area.

Sample	No.	Colour	pH	Temperature
1		Black	7	31˚C
2		Brown	7	31˚C
3		Black	7	30˚C
4		Brown	7	31˚C
5		Black	7	30˚C

Table 3
Colonial characteristics of the Actinomycetes isolates on Starch casein agar (SCA).

Isolate No.	Incubation period	Colony Morphology	Figure of Gram staining
D3	4-5 days	Intermediate, Round, Smooth, Wavy, Flat, Opaque, White colony.	
S7	5-7 days	Intermediate, Round, Smooth, Entire, Low convex, Opaque, White colony	
W7	4-5 days	Large, Round, Smooth, Entire, Convex, Translucent, White colony	
W15	4-5 days	Intermediate, Round, Smooth, Entire, Convex, Opaque, Chalk white colony	
W19	4-5 days	Large, Round, Rough, Wavy, Convex, Opaque, Chalk white colony	
W20	4-5 days	Intermediate, Round, Smooth, Wavy, Flat, Translucent, Light yellow colony	

All the isolates were biochemically studied and characterized (Table 4). All the isolates revealed positive response over catalase test. However none of the isolates showed positive reaction for, H_2S production test and Urea utilization test. Out of 6 isolates of Actinomycetes, 3 isolate shows positive reaction for starch hydrolysis test.

Table 4
Biochemical characterization of marine Actinomycetes isolates.

Isolate No.	Hydrogen sulphide	Starch Hydrolysis	Urea utilization	Catalase test
D3	–	+	–	+
S7	–	+	–	+
W7	–	+	–	+
W15	–	–	–	+
W19	–	–	–	+
W20	–	–	–	+

(–) Negative; (+) Positive

The carbohydrate utilization tests by isolates shows different pattern with different carbon sources (Table 5). Except one isolate S7 all the isolates utilized the Lactose. Four isolates including S7, W7, W19 and W20 utilized Mannitol. However, Sucrose was utilized by S7, W7, and W19 isolates. Other carbohydrate source like Maltose not utilized by D3 and W20 isolates and Xylose also not utilized by D3 and W19 isolates.

Table 5
Utilization of carbon source by Actinomycetes isolates.

Isolate No.	Sucrose	Lactose	Mannitol	Maltose	Xylose
D3	–	+	–	–	–
S7	+	–	+	+	+
W7	+	+	+	+	+
W15	–	+	–	+	+
W19	+	+	+	+	–
W20	–	+	+	–	+

(–) Negative; (+) Positive

Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity.[12] Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidant.[13] In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound (Figure 1). Presence of reducers causes the conversion of the Fe^{+3}/ferricyanide complex used in this method to the ferrous (Fe^{+2}) form. By measuring the formation of Pearl's Prussian blue at 700nm, it is possible to determine the concentration of Fe^{+3}ion.[14]

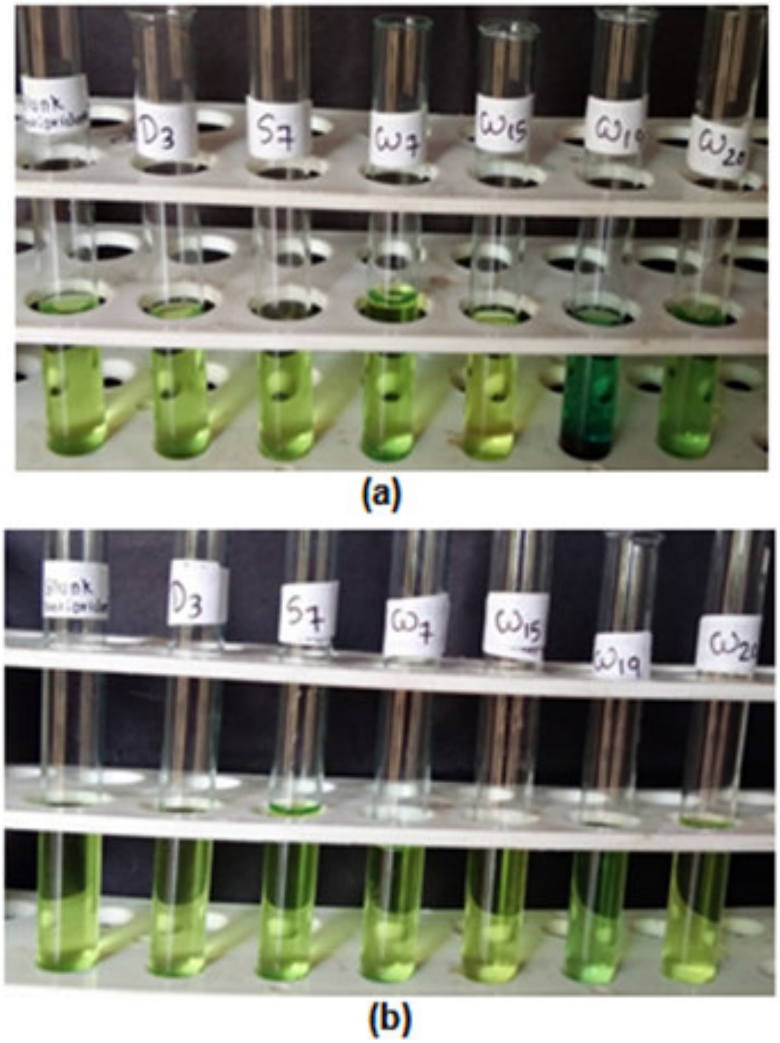
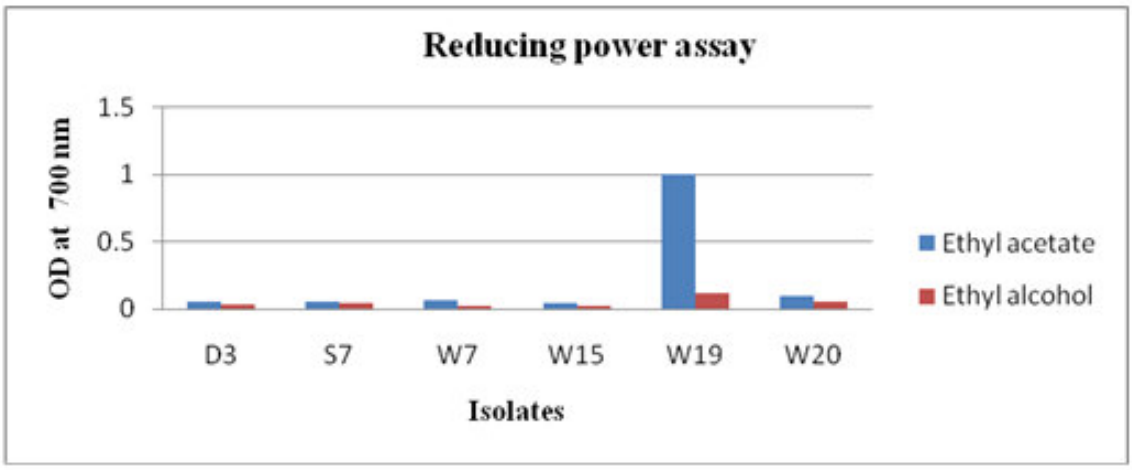


Figure 1
Reducing power activity of metabolites from Ethyl acetate extracts and Ethyl alcohol extracts.

Metabolites of ethyl acetate extract have greater reducing power than metabolites of ethyl alcohol extract. Actinomycetes isolate W19 shows highest reducing power than isolates D3, S7, W7, W15, W19 and W20. (a) Reducing power activity of metabolites from ethyl acetate extracts, (b) Reducing power activity of metabolites from ethyl alcohol extract. Metabolites of ethyl alcohol extracts from test samples showed slightly lower reducing power than metabolites of ethyl acetate extracts from isolated Actinomycetes. The reducing power of the metabolites from ethyl acetate extracts and ethyl alcohol extracts of the isolated. Reducing power of Actinomycetes are shown in Graph 1. The maximum Reducing power was given by metabolites of ethyl acetate extracts and ethyl alcohol extracts of the Actinomycetes isolate W19 with absorbance of 1.0 and 0.12 at concentration 0f 100µg/ml respectively. As shown in Graph 1, a higher absorbance value indicates a stronger reducing power of the samples.

Graph 1
Reducing power activity of metabolites from ethyl acetate and ethyl alcohol extract of Actinomycete isolates.



Metabolites of Actinomycetes isolate W19 have highest reducing power of about 1.0 and 0.12 at the concentration of 100µg/ml respectively.

Cytotoxic activity assay

The cytotoxic effects of extracted metabolites from the various isolates on the growth of eukaryotic cells were studied. For the cytotoxic activity studies yeast *Saccharomyces cerevisiae* cells were chosen as a model eukaryotic organism. *Saccharomyces cerevisiae* cells were exposed to various concentrations of the extracted metabolites and cell viability evaluated by measuring absorbance after 2h of exposure (Graph 2). The cytotoxicity effects of the metabolites from ethyl acetate extracts were increased with the increase of the metabolites concentration. At metabolites concentration of (50µl) cell viability greatly reduced by 74.4% after 2h of exposure of W19 isolate. The metabolites of isolate W19 shows highest cytotoxic activity of about 74.4% of inhibition, isolate W7 shows 63.6% of inhibition, isolate W20 shows 61.6% of inhibition, isolate S7 shows 58.4% of inhibition, isolate W15 shows 51.2% of inhibition and isolate D3 shows lowest inhibition of about 48% These results suggested that crude compound from isolated Actinomycetes was toxic to *Saccharomyces cerevisiae* cells.

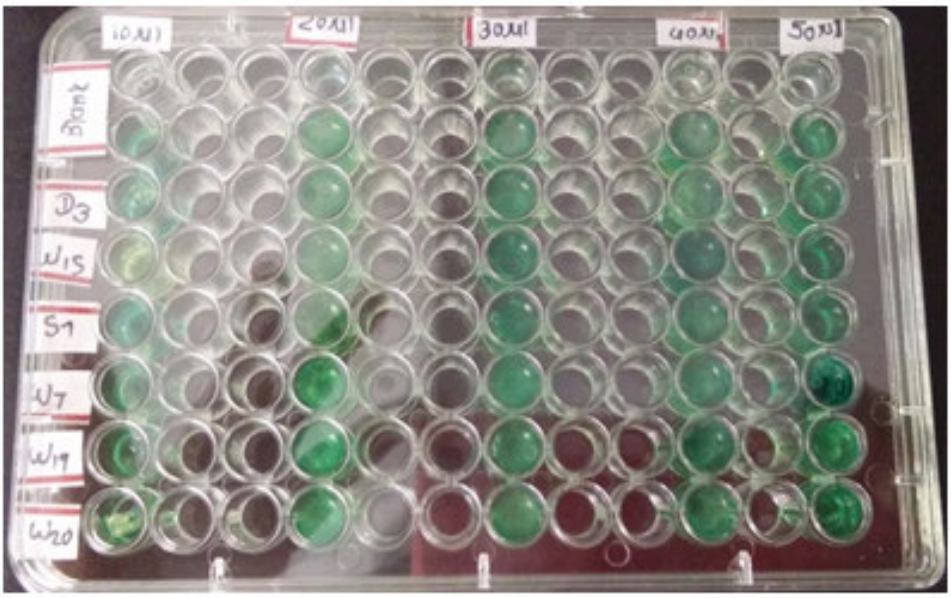
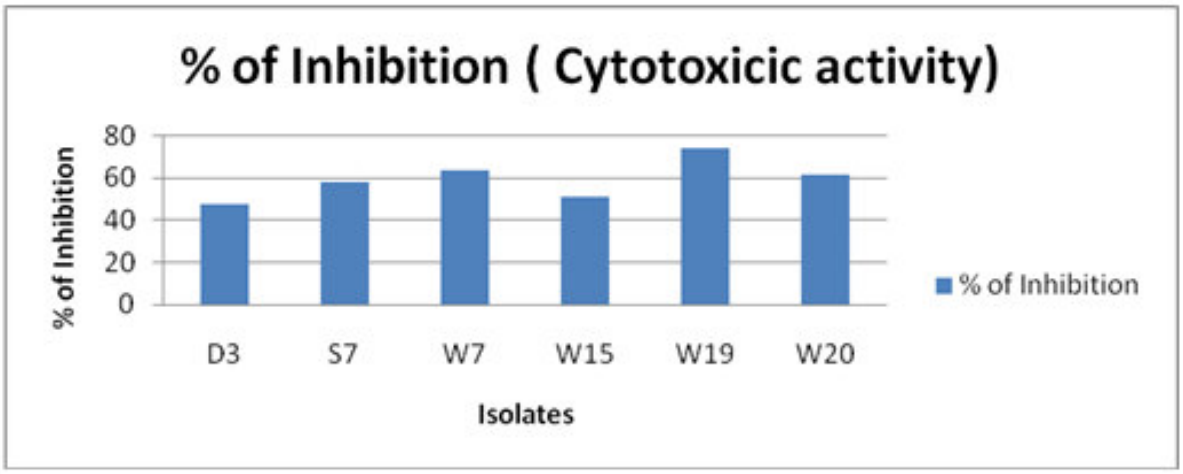


Figure shows cytotoxic activities of metabolites from ethyl acetate extract. Actinomycetes isolate W19 shows about 74.4% of cell inhibition which is highest inhibition, isolate W7 shows 63.6%, isolate W20 shows 61.6%, isolate S7 shows 58.4%, isolate W15 shows 51.2% of inhibition, while isolate D3 shows lowest cell inhibition of about 48%.

Figure 2
Cytotoxic activity of metabolites from ethyl acetate extracts

Graph 2
Cytotoxic activities of metabolites from ethyl acetate extract of isolated Actinomycetes on Saccharomyces cerevisiae cells.



Metabolites of Actinomycetes isolate W19 have highest cytotoxic activity of about 74.4% of cell inhibition with Ic50 7.46µg/ml.

The cytotoxicity effects of the metabolites from ethyl acetate extracts were increased with the increase of the metabolites concentration. At metabolites concentration of (50µl) cell viability greatly reduced by 74.4% after 2h of exposure of W19 isolate. The metabolites of isolate W19 shows highest cytotoxic activity of about 74.4% of inhibition, isolate W7 shows 63.6% of inhibition, isolate W20 shows 61.6% of inhibition, isolate S7 shows 58.4% of inhibition, isolate W15 shows 51.2% of inhibition and isolate D3 shows lowest inhibition of about 48% (Graph 2). These results suggested that crude compound from isolated Actinomycetes was toxic to *Saccharomyces cerevisiae* cells.

DISCUSSIONS

Microorganisms are attractive source of biologically active compounds having pharmaceutical and agricultural significance. These Actinomycetes are biotechnologically and industrially valuable prokaryotes as they produce a large number of bioactive compounds with pharmaceutical and agricultural importance.[15] In the present study, we have isolated 6 Actinomycetes from a marine soil and water sample from Dholai Bandar area of Valsad district, Gujarat, India. The isolates were characterized as an Actinomycetes on the basis of microscopic, morphological and biochemical characteristics. This study evaluated the Antioxidant and Cytotoxic activity of the secondary metabolites of Actinomycetes. The secondary metabolites from the crude extract of marine Actinomycetes isolates shows remarkable antioxidant activity. In recent years much attention has been devoted to natural antioxidant and their association with health benefits.[16] There are several methods available to assess antioxidant activity of compounds. An easy and rapid method for the antioxidant screening is reducing power assay. The assay is performed in order to measure the reducing power of the compounds. In the present study, we investigated the $Fe^{+3} \rightarrow Fe^{+2}$ transformations in the presence of extracted metabolites from the isolated Actinomycetes.[17] In this assay, the reductants (antioxidant) would cause the reduction of Fe^{+3} to Fe^{+2} by donating an electron. The amount of Fe^{+2} complex formed can be monitored by measuring the formation of Perl's Prussian blue at 700nm.[18] The reducing capacity of a compound may serve as a significant indicator of metabolites compounds potential antioxidant activity.[19] In this study, the reducing power of metabolites extract with ethyl acetate solvent was higher than the metabolites extract with ethyl alcohol solvent. Similar results have been derived by Hanane El Hajaji *et al.,* (2010)[20] . The metabolites from ethyl acetate extract and ethyl alcohol extract of W19 isolate was found to possess higher reducing power of about 1.0 and 0.12 at the concentration of 100µg/ml respectively, it is evident that isolate W19 metabolites possess very good reductive potential and could serve as electron donors, terminating the radical chain reaction.[18] Similar results have been observed in previous studies of Vanmathi K. *et al.,* (2016), where compound isolated from marine Actinomycetes *Streptomycetes species* ABTRI 1 strain has been showed maximum reducing power 0.16 at the concentration of 100µg/ml.[20] Antioxidant and Antipoliferative potentials of marine Actinomycetes were also studied in which the total antioxidant power of the crude extracted metabolites of the isolated Actinomycetes increased with increasing concentration of metabolites.[22] It was observed that potent isolate NSRB from salterns soil samples from Kothapattanam, Ongole, Andhra Pradesh showed reducing power 0.59%.[23] In present study the metabolites of isolate W19 shows 74.4% of growth inhibition which is strongest growth inhibition against *Saccharomyces cerevisiae* cells at a density of 1×10^8 and for isolate W7 63.6% of inhibition, for isolate W20 61.6% of inhibition, for isolate S7 58.4% of inhibition, for isolate W15 51.2% of inhibition and for isolate D3 48% of inhibition which is lowest growth inhibition compare to other isolates. Similar study have been carried out and observed in HeLa cell line, where the extract of isolate 2 shows about 68% of inhibition which is strongest growth inhibition, 61% inhibition for isolate 1 and 59% inhibition for isolate 3 in human cervical cancer cell line (HeLa).[7] In the last decades, many studies have determined the anticancer activity of Actinobacteria isolated from marine

environments.[24] The cytotoxicity of bioactive isolates observed in this study was similar to the one who found that the cytotoxicity effects of marine *Streptomyces* extracts were increases with the increase of the extracts concentration.[25]

CONCLUSION

The study was successfully done with the isolation and determination of antagonistic Actinomycetes from marine soil and water sample of Dholai Bandar and extracted metabolite from ethyl acetate solvent of isolated Actinomycetes shows effective biological activities such as antioxidant and cytotoxic activities. The result of the present study highlighted that marine Actinomycetes can be used as the potential source of natural antioxidant and cytotoxic compound, in the pharmaceutical and medical industries or as a food supplement. Further, work should be done to evaluate the isolation and identifications of the antioxidant and cytotoxic component of the extracted metabolites by various different analytical methods.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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Dr.M.Muthuraj

Bacteriologist
Govt. Hospital for Chest Diseases, Gorimedu, Pondicherry.

Dr Hetal K.Panchal,M.Sc.,Ph.D

Asst.Prof,Microbiology Dept,Environmental industry microbiology,Dolat-Usha Institute of Allied Science and Dhiru-Sarla Institute of Management & Commerce,College

G. Bakhya Shree M.S. (Research)

Coordinator and Trainer, Department of Biotechnology and Life Sciences, Dexter Academy, Madurai, Tamilnadu

Prof.Dr.K.Suriaprabha

Asst. Editor , International Journal of Pharma and Bio sciences.

Prof.P.Muthuprasanna

Managing Editor , International Journal of Pharma and Bio sciences.

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