Evaluation Of In Vitro Anticancer Activity of Crude Extracts Obtained from Two Bacterial Strains Isolated from Kodiyampalayam, Tamil Nadu

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Abstract: ***Extracts of natural products, especially microorganisms, have been a valuable source of various molecules in many drug discovery efforts and led to the discovery of many important drugs. The identification of microbial strains with promising biological activities and the purification of biomolecules responsible for activities led to the discovery of many biologically active molecules.Crude extracts of two marine bacterial isolates isolated from marine sediment samples, were studied for their in vitro anticancer activity against human oral squamous carcinoma (KB) cell line. Morphological studies and biochemical tests of the two bacterial extracts were also carried out. Cytotoxic, intracellular ROS, nuclear staining and apoptotic morphological alteration studies were carried out to assess the anticancer potential of each extract. The crude ethyl acetate extract of KP-9 isolate showed promising results by MTT assay with IC50 as low as 7.9 µg/ml while as KP-7 showed an IC50 value of 21.1µg/ml in KB cells. The crude extract of KP-9 augmented higher levels of ROS and displayed higher potential by inducing higher levels of nuclear and morphological alterations when compared with KP-7 bacterial extract in KB cells.***

Keywords: ***Anticancer activity, Microbial extracts, Cytotoxicity, ROS.***

I. INTRODUCTION

Over the past 75 years, compounds derived from natural products have led to the discovery of many drugs for the treatment of many human diseases [17]. Natural products are chemical compounds derived from organisms, for example plants, animals and microorganisms. Can be defined as chemical compounds isolated or derived from organisms as primary or secondary metabolites. Through the use of advanced techniques in many detection programs, the detection rate of natural compounds exceeded one million sofa [30]. Of which 22,500 are biologically active compounds extracted from microbes, 45% are

produced by actinobacter, 38% by fungi and 17% by unicellular bacteria [10]. The oceans cover more than 70% of the Earth's surface, and little is known about the microbial diversity of marine sediments, an inexhaustible resource that has not been fully exploited. Severe marine organisms act as a valuable natural source of new products such as antibiotics, antineoplastic agents and other therapeutic substances [2].Secondary microbial metabolites are known as one of the enormous reservoirs of natural chemical diversity with active biological activity [6].

Most bacterial secondary metabolites are created by a unique multi-step biological process with specific enzymes for each complex structure formation. Their coding genes are usually assembled within the organism's genome and biosynthesis precursors are obtained from primary metabolites.

Research on natural biologically active compounds that can act as antioxidant and anti-cancer agents has gained interest in industry and in scientific research. The harmful nature of reactive oxygen species (ROS) produced during oxidation processes, and the harmful nature of synthetic antioxidants such as butyl hydroxyanol (BHA) and butyl hydroxytoloin (BHT) and increased microorganism resistance to synthetic drugs have contributed to the increased search for new antibiotics. Biologically active ingredients of natural origin [29].

The escalating knowledge of reactive oxygen species (ROS) has revolutionized medicine [26]. ROS contributes to cardiovascular diseases and cancer [3,18] and could cause immune system depletion [18,28]. A typical antioxidant donates an electron to a free radical thus neutralizing it [19] and often bind to metals [5].Synthetic antioxidants cause negative health effects [11]. Reports have recommended to replace synthetic antioxidants with natural ones [5,32] to control formation of free radicals [8]. The most common natural antioxidants are antioxidative enzymes [16] whereas others are well represented in different spices and herbs [20].

From pulmonary disorders to autoimmune diseases to cancer (the focus of this review), MMPs have been found to directly contribute to disease progression [12,31]. MMPs are present in nearly all human cancers; they can be expressed by healthy fibroblasts in the adjacent stroma, cancer-associated fibroblasts, and/or by non-fibroblastic cancer cells. This is of great significance, as MMPs can influence the tumor environment by promoting angiogenesis, tumor growth, and

Revised Manuscript Received on October 25, 2019.

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metastasis.[4,22].Accordingly, MMP expression is tied to tumor aggressiveness, stage, and patient prognosis [21,37].

Transcription of MMPs is tightly regulated and expression is generally very low. Further regulation of MMP activity occurs by post-translational modification, production of the enzymes as zymogens requiring activation, and coexpression of tissue inhibitors of metalloproteinases (TIMPs)[7,34].Dysregulation of any of these regulatory mechanisms during pathological conditions may contribute to worsening of disease[34]. Increased expression of MMPs is correlated to increased cancer cell proliferation and an increase in tumor size. Overexpression of MMP-3 in the phenotypically normal murine mammary epithelial cell line, SCp2, using a tetracycline inducible system injected into surgically cleared mammary tissue has been shown to be sufficient to induce spontaneous disease progression [33].Similarly, MMP-2 levels detected in cancer tissue are significantly correlated to larger tumor size [25].Several MMPs have been shown to drive cell migration and invasion through the basement membrane. Sequence-specific silencing of MMP-14 alone significantly attenuates both migration and invasion of cancer cells in vitro[35].

II. MATERIALS AND METHODS

A. Isolation of bacterial strains

Samples were taken at the depth of 2 cm inside the marine sediment. Samples were collected in sterile polypropylene tubes and immediately transferred to the lab for further analysis. The soil samples were suspended in sterile autoclaved nutrient broth and incubated at 37 °C overnight. The broth culture was

serially diluted with sterile phosphate buffered saline (PBS) and plated on nutrient agar. The plates were then incubated for 24 h at 37 °C. The pure colonies of each isolate were then isolated and used for further analysis.

B.Cell morphology and biochemical tests

The morphological and biochemical characterization was carried out for the two bacterial strains (KP-7 and KP-9). The morphological characteristics like colony colour, shape and forming of the bacterial strains was studied by Grams staining and microscopic examinations of each strain. The biochemical characterization studies of each strain (KP-7 and KP-9) included indole test, methyl red test, Voges–Proskauer (VP) test, Citrate reduction test, Triple Sugar Iron (TSI)test, catalase and oxidase test was carried out.

C. Cell line and culture

The human oral squamous carcinoma (KB) cell line was obtained from cell repository of National Centre for Cell Sciences, (NCCS) Pune, India. KB cells were cultured in Dolbico's Eagle's minimal essential medium (DMEM, Himedia) with 2.0 mM of L-glutamine, 1.5 g/l of $NaHCO_3$, 0.1 mM of nonessential amino acids, 1.0 mM of sodium pyruvate, and supplemented to contain 10% (v/v) fetal bovine serum (FBS, Himedia). Cells were grown at 37 °C and 5% CO_2 in humidified air.

D. MTT assay

A total of 106 $x10^7$ cells were seeded into 96-well plates and exposed to different bacterial extracts for 1 hour. The cytotoxicity of the bacterial extracts was assessed by the MTT assay according to the procedure used by Igarashi and Miyazawa [18]. MTT (50 µl) was added to the treated wells. The plate was incubated at 37 °C for 3 hours with mild shaking. The cells were then resuspended in 200 µL of DMSO for 30 min in the dark. The absorbance was read at 650 nm, in a microtiter plate reader (Anthos, Germany).

E. Determination of ROS by DCFH-DA dye method

Intracellular ROS was determined by the method of [23]. KB cells (1x 10^4 cells) were seeded in 96-well plate and exposed to different concentrations (1, 5 and 10 µg/ml) of bacterial extract (50, 100, and 200 m M) for 24 h. After exposure, cells were incubated with 2,7-dichlorodihydro fluorescein diacetate (DCFH-DA) (10 mM) for 30 min at 37 °C. The cells were washed twice with PBS and resuspended in 1 ml of PBS. Fluorescence intensity was measured (excitation/emission at 495 nm/529 nm). Cells were also observed under fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

F. Determination of apoptosis by AO/EtBr dual staining

AO/EtBr method was carried to determine the apoptosis according to the method of [9]. KB cells were seeded in a 12-well plate for overnight at 37 °C. The cells were then treated with different concentrations (1, 5 and 10 µg/ml) of bacterial extracts for 24 h. The treated cells were then incubated for 60 min with 10 µL of EtBr (5 mg/mL). The cells were washed twice with PBS and resuspended in PBS. Cells were then observed for apoptosis under a fluorescence microscope (Nikon, Japan) (400 X) magnification. The ratios of apoptotic cells to normal cells were calculated and expressed as percentage apoptosis for each treatment group.

G. DAPI assay

The apoptotic effect of the bacterial extracts was analyzed by fluorescent nuclear dye DAPI according to the method of [1]. KB cells were seeded in a 12-well plate and treated with different concentrations (1, 5 and 10 µg/ml) of bacterial extracts for 24 h. Cells were then stained with DAPI dye for 1 hour, washed twice with PBS and observed under blue filter on a fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

H. Statistical analysis

Each experiment was performed at least six times (n = 6) and the results are presented as the mean ± SD. The statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA). Graphs are prepared using Microsoft Office Excel 2007. The IC_{50} values were calculated using the linear regression analysis.

III. RESULTS

A. Morphological and biochemical characterization of isolates

The morphological and biochemical characterization was carried out for the two bacterial strains (KP-7 and KP-9). The results are given in Figure 1 and Figure 2; Table. 1.

Figure1. Isolation of two isolates (KP-7 and KP-9) from marine sediment samples.

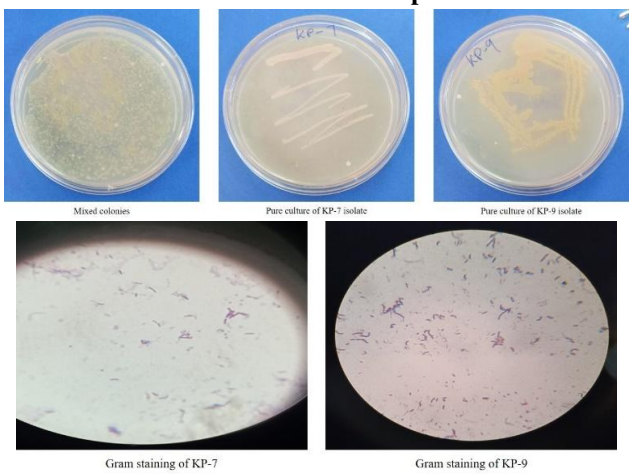

Figure 2. Isolation of two isolates (KP-7 and KP-9) from marine sediment samples.

Table 1: Biochemical and morphological characteristics of the bacterial strains (KP-7 and KP-9) isolated from the marinesamples.

Biochemical test	KP-7	KP-9
Indole	-	-
Methyl Red	-	-
VP	-	-
Citrate	+	+
Triple Sugar iron	Acid bud Alkaline slant	Acid bud Alkaline slant
Catalase	-	+
Oxidase	-	+
Morphological characteristics		
Colony colour	White	Yellow
Cell morphology	Rod shaped	Rod shaped
Forming	Chain	Chain

B.Effect of bacterial extracts on cell viability of KB cells

We observed a considerable decrease in the cell viability of KB cells when treated with different concentrations of bacterial extracts. However, cell viability was found to be decreased more in the cells treated with extract of KP-9 bacterial strain. The IC_{50} values of KP-7 and KP-9 bacterial extracts were measured to be 21.1 µg/ml and 7.9 µg/ml, respectively (Figure. 3). The results obtained from this assay clearly validate that the extracts obtained from KP-7 and KP-9 bacterial strains possess excellent cytotoxic activity against KB cells.

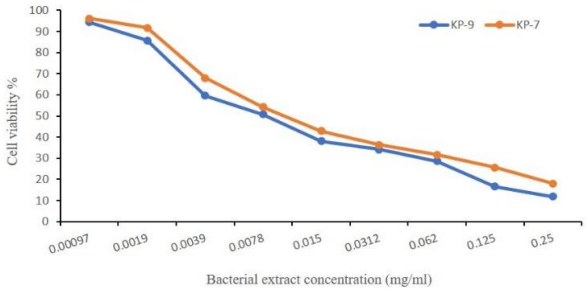

Figure. 3. Cytotoxicity of extracts from two different bacterial strains (KP-7 and KP-9) in KB cells. Cells were treated with different concentrations of KP-7 and KP-9 bacterial extracts for 24 h at 37 °C. Cell viability was determined, and results were given as mean and ± SD of six separate experiments.

C.Effect of bacterial extracts on intracellular ROS in KB cells

We observed a significant increase in the intracellular ROS formation in KB cells when treated with different concentrations of bacterial extracts (Figure. 4). The KP-9 bacterial extract was profoundly able to induce higher levels of intracellular ROS in KB cells when compared to treatment with KP-7 bacterial extract. The increase in the intracellular ROS in KB cells followed a concentration dependent manner. The results obtained from this assay prove that the KP-7 and KP-9 bacterial extracts have the potential to induce the formation of intracellular ROS in KB cells.

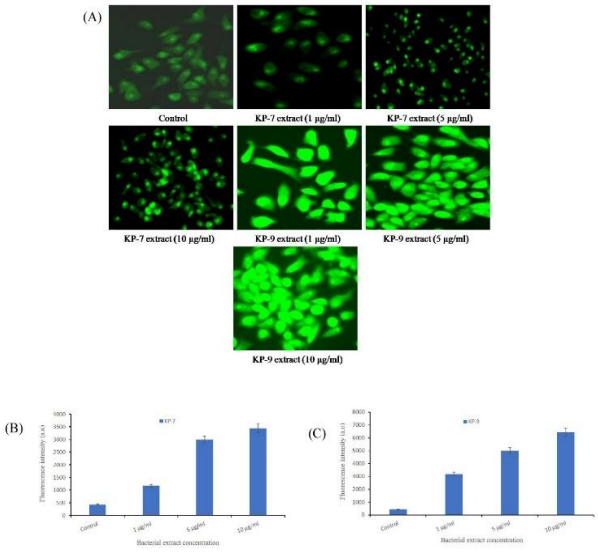

Figure. 4. effect of extracts from two different bacterial strains (KP-7 and KP-9) on intracellular ROS generation in KB cells. Cells were treated with different concentrations of KP-7 and KP-9 bacterial extracts for 24 h at 37 °C. Cells were observed (A) microscopically and for fluorescence intensity under green filter (B) and (C). Results are given as mean and ± SD of six separate experiments.

D. Effect of bacterial extracts on cellular apoptosis in KB cells

We found that the KP-7 and KP-9 bacterial extract treatments were able to induce irregular nuclear morphology in KB cells which is a hallmark of cell apoptosis.

The levels of apoptosis followed a concentration dependent manner in KB cells. While the KP-9 bacterial extract induced higher levels of cellular apoptosis, mild levels of apoptosis were observed with the increased concentrations of KP-7 bacterial extract treatments in KB cells (Figure. 5).

E. Effect of bacterial extracts on the nuclear alterations in KB cells

We observed that the KP-7 and KP-9 bacterial extracts treatments induced significant levels of nuclear alterations in KB cells. Treatment with extract obtained from KP-9 showed more potential to induce nuclear alterations in KB cells when compared to the KP-7 bacterial extract treatment (Figure. 6). Our results from this assay show that extracts obtained from KP-9 and KP-7 bacterial strains possess efficient potential to induce nuclear alterations in KB cells.

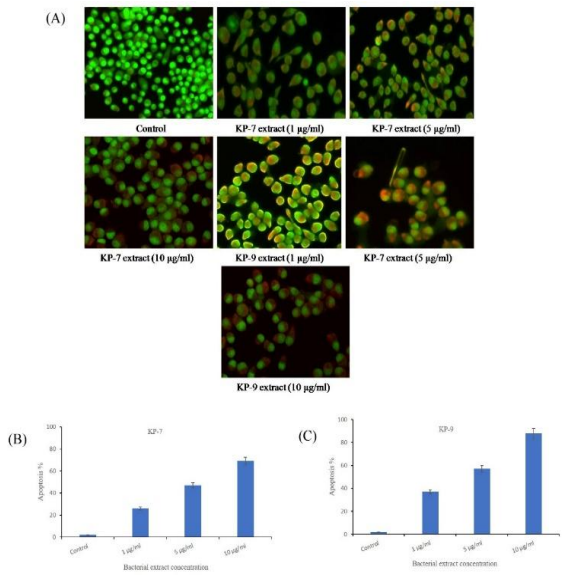

Figure. 5. Effect of bacterial extracts (KP-7 and KP-9) on apoptosis in KB-cells. Cells were treated with different concentrations of KP-7 and KP-9 bacterial extracts for 24 h at 37 °C. Cells were observed (A) microscopically and for fluorescence intensity (B) and (C) under blue filter. Results are given as mean and ± SD of six separate experiments.

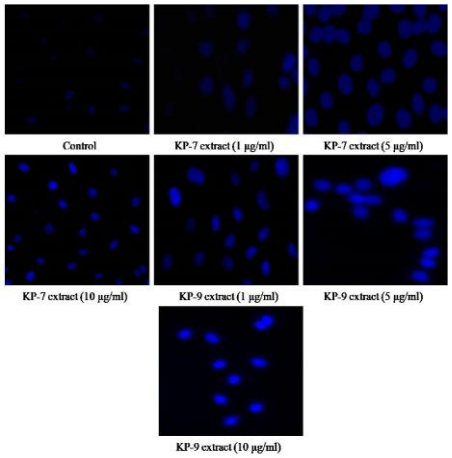

Figure. 6. Effect of bacterial extracts (KP-7 and KP-9) on nuclear alterations in KB-cells. Cells were treated with different concentrations of KP-7 and KP-9 bacterial extracts for 24 h at 37 °C. Cells were observed (A) microscopically and for fluorescence intensity (B) and (C) under blue filter. Results are given as mean and ± SD of six separate experiments.

IV. DISCUSSIONS

Our study represents the first anticancer report of the crude extract of two bacterial strains isolated from the marine sediments. The results from the current study showed that the crude extracts of KP-7 and KP-9, exhibited potential anticancer activity in KB cells. KP-9 bacterial extract showed more potential activity when compared to KP-7 bacterial strain. By combining the knowledge of different anticancer assays and assessment of anticancer parameters in the present study, it can be asserted that the investigated bacterial strains (KP-7 and KP-9) could be viable source for natural anticancer agents. The current study also provided evidence that the extracts of the two bacterial strains are the source of natural therapeutic agents, which can be accounted for the traditional uses in the prevention of cancer and health preservation. This study provides supportive data for future investigations that will lead to their use in cancer therapy. Purification of the active components present in the crude extract of the two bacterial extracts and detailed pathway analysis could be performed to understand their basic mechanism of action. The reliable criteria for judging the value of anticancer drugs are prolongation of lifespan, decrease of WBC from blood and decrease of tumor volume. The reduction in cell viability showed that the crude extracts plays a direct role in killing tumor cells and enhance the curative effect tumor chemotherapy. At high concentration, ROS can produce oxidative damage, especially in the DNA, causing cell death [27]. The present study showed that crude extracts significantly elevated intracellular ROS levels which followed a concentration dependent manner. While the KP-7 bacterial crude extract exhibited a mild activity but KP-9 bacterial extract displayed significant activity in KB cells.

This theory forges the fundamental link between neoplasia and apoptosis, as exemplified by the ability of oncogenes including Myc, and tumor suppressors such as p53 to actively engage apoptosis.The ability of Myc to drive apoptosis in addition to providing a potent proliferative signal is interpreted as a failsafe mechanism to offset its oncogenic capacity [24]. Treatment with crude extracts significantly induced the apoptosis in KB cells. The KP-9 bacterial extract was noticed to be inducing more apoptosis when compared to KP-7 bacterial crude extract. This showed that bacterial extracts possess activity against proliferation of KB cells. Normal cells comprise an intact cell nucleus with a nonrandom spatial organization of chromosomes and genes. Such cell type-specific architecture can be altered in various diseases through impaired cell microenvironmental interactions leading to globulated and indented nuclear morphologies with defective nucleoli and other nuclear bodies [36] Increased nuclear alterations with bacterial (KP-7 and KP-9) crude extract treatments caused alterations in the nuclei and followed a concentration dependent fashion.

V. CONCLUSION

We propose that the crude extract of two bacterial extracts, KP-7 and KP-9, have significant antitumor activity. The present study demonstrates that the crude extract of KP-7 and KP-9 decreased the cell viability, increased the intracellular ROS levels, induced apoptosis and increased nuclear alterations in KB cells. The data suggest that crude extract of KP-7 and KP-9 possesses anticancer properties valuable for application in drug development strategies.

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