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Biochemical characterization and anticancer activity of newly isolated haloalkaliphilic bacterial strain against hepatocellular carcinoma *in vitro*

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Abstract:

Incidence and mortality rates of liver cancer vary widely geographically, making it the sixth most common cancer in the world. Microorganisms that grow in high-salinity environments can produce bioactive compounds for therapeutic applications in the pharmaceutical field. In this study, a bacterial strain, Marinobacter HA2, was isolated from El-Hamra Lake in Wadi El-Natrun, Egypt. The biochemical features and anticancer activity were investigated. The cytotoxicity of the crude extract was assessed using the HepG2 cell line and normal human peripheral blood lymphocytes (HPBL) *in vitro*. The IC_{50} of the crude extract using the MTT test on the HepG2 cell line was calculated at 82±1.4 µg/ml.

Keywords: Liver cancer; haloalkaliphilic bacteria; human peripheral blood lymphocytes; *Marinobacter HA2*; Wadi El-Natrun; HepG2.

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1. Introduction:

Liver cancer is still a major global health concern, and its prevalence is rising globally (Llovet et al., 2016; Villanueva et al., 2019). Hepatocellular carcinoma (HCC) makes up around 90% of all occurrences of liver cancer (Akinyemiju et al., 2017). Genetic and epigenetic alterations accumulate during cancer, which is a multi-stage disease. These changes result in the inactivation of tumor suppressor genes and the dominant activation(Akinyemiju et al., 2017, Yameny, A. 2017).

of several oncogenes, which ultimately cause healthy cells to convert malignantly (Bray et al., 2018). Cancer treatment may involve surgery, radiation, chemotherapy, or targeted therapy. Side effects include immunodeficiency, cell damage, and neurological, renal, and cardiac toxicity; therefore, new cost-effective strategies and therapies to mitigate these side effects are needed (Nachega et al., 2011). Haloalkaliphilic bacteria are adapted to grow under extreme environments of salinity and alkaline pH (Sorokin et al., 2008). Halophilic microorganisms are promising candidates and a hope for drug discovery because of their high

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metabolic versatility, low nutritional needs, and genetic machinery that adapts to harsh conditions like nutrient starvation, desiccation, high sun radiation, and high ionic strength (Charlesworth & Burns, 2015). An important source of anticancer drugs is natural products, sometimes called bioactive molecules. Although the earlier and well-established anticancer natural products were derived from plant cells, microorganisms are an excellent alternative due to their diversity. Traditionally, the most conventional method of inhibiting cancer cell viability has been with the use of bacterial metabolites. Extremophiles are increasingly being studied as a source of novel biomolecules today (Wang et al., 2019). In Egypt's Wadi El-Natrun, 90 km northwest of Cairo, a chain of seven large alkaline and hypersaline lakes runs through the valley. The lakes are fed by underground seepage of water from the Nile River and are characterized by a high evaporation rate, pH values (8.5–9.5), and salinities (283–290) (El-Ghani et al., 2015). These challenging environments are a rich source of microbes with potentially valuable biological activities. The unique aquatic ecosystem of such lakes is characterized by high levels of sulfate, chloride, carbonates, sodium, and trace magnesium (Zheng & Liu, 2009). Halophilic and alkaliphilic microorganisms, including archaea, purple bacteria, and cyanobacteria, thrive in these lakes, creating red, purple, and green waters (Imhoff, 2017).

In the current work, we focused on the biochemical characterization and anticancer potential of extracellular metabolites on the HepG2 cell line produced by halophilic bacteria (*Marinobacter HA2*) isolated from El-Hamra Lake, Wadi El-Natrun, Egypt.

2. Materials and Methods:

2.1 Bacterial strain collection and identification:

The bacterial strain (*Marinobacter HA2*) with accession number KU323642 in GenBank was derived from the Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat, Egypt.

2.2 Biochemical characterization of *Marinobacter HA2*

2.2.1 Motility test

Two drops (about 0.1 ml) of sterile distilled water were added to a sterile tube to create a cell suspension. One drop of bacterial suspension was transferred onto a microscope slide and overlaid with a glass cover then examined under a light microscope (400X) (Leffert et al., 1970).

2.2.2 Catalase test:

The catalase test was investigated for the derived strain as described before (Clarke & Cowan, 1952). The catalase enzyme converts hydrogen peroxide to oxygen and water. In that test, a drop of 3% hydrogen peroxide was added to a microbial colony that had been smeared on a sterile glass slide. The positive catalase reaction was demonstrated by the foam that formed and the released bubbles that emerged from the oxygen.

2.2.3 Oxidase test:

The oxidase test reaction was conducted using a Dry slide oxidase test kit (Difco, USA) according to manufacturer kit protocol. Following the protocol, the bacterial smear was observed for positive oxidase reaction after 2 min. The test was positive when a dark purple color developed.

2.2.4 Starch hydrolysis:

Starch degradation (l % w/V) was detected in Petri dishes containing a starch nitrate medium and inoculated with the tested organism. The plates were floated with an iodine solution after 2 days of incubation at 30 °C for starch degradation (Cowan, 1974).

2.2.5 Urea hydrolysis test:

The rapid determination of urea hydrolysis for the urease test was conducted as described before (Qadri

et al., 1984). Urea agar base (Difco, USA) was ten-fold diluted and adjusted at pH 6.95. The bacterial culture was adjusted at a fixed density of McFarland standard of 3. The test tubes were covered with parafilm and incubated at 35 °C for color detection at 5-, 15-, 30-, 60-, and 120-min intervals.

2.3 Anticancer activity of bacterial secondary metabolites:

2.3.1 Preparation of crude extract:

The bacterial isolate was cultured in the same broth medium and incubated in a shaking incubator at 100 rpm at 30 °C for 72 h. The culture medium was centrifuged at 6000 rpm for 20 min, and the supernatant was collected and filtered through 0.45 µm sterile membranes. Culture filtrate was mixed with ethyl acetate (1:1) and stirred at 130 rpm for 12 h. The mixture was poured into a separating funnel, and the ethyl acetate phase was removed and followed by vacuum evaporation to obtain the dry extracts by vacuum rotary evaporator (Rotavapor, Heidolph, Schwabach, Germany) at 40 °C (Thomas et al., 2011). The produced crude extract was stored at -20 °C for further investigations.

2.3.2 Biological investigations:

In vitro, the extract's impact was assessed against the normal human peripheral lymphocytes (HPBL) and the hepatocellular carcinoma (HepG2) cell line. The study was authorized and complied with the rules set by the Institutional Ethical Committee guidelines at Faculty of Science, Menoufia University, Egypt.

2.3.3 Maintenance of HepG2 cell line:

The cell line for hepatocellular carcinoma (HepG2) was purchased from VACSERA in Giza, Egypt. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin was used to maintain and cultivate the cell line. Cells were cultivated at a density of 2×10^5 cells/ml in T25 culture flasks at 37 °C in a humidified 5% CO2 environment. Every 48 hours, the medium in the flasks was replaced. An inverted microscope was used to confirm the cells' confluency up to 75%. Following trypsinization (0.025% trypsin and 0.02% EDTA), cells were extracted and then given two PBS washes. A hemocytometer was used to measure the concentration of the cell suspension per milliliter, and the following formula was used to compute it:

$$\text{Cells/ml} = 10^4 \times (\text{Average count per square}) \times (\text{Dilution factor})$$

All experiments were conducted in triplicate. All reagents and media were sourced from Lonza in Egypt.

2.3.4 Cytotoxicity on HepG2 cell line:

To evaluate the maximal half inhibitory concentration (IC_{50}), cytotoxicity of *M.* HA02 bacterial extract was carried out by microculture tetrazolium (MTT) assay method (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) against HepG2 cell line. Briefly, cells were seeded into 96-well plates at a plating density of 1×10^4 cells/well and incubated to allow the attachment of cells before the addition of the treatments. The bacterial extract was dissolved in dimethyl sulfoxide (DMSO) and diluted in a serum-free medium. After reaching the confluency of 75%, serial dilutions of the extract were added to the cultures and then incubated for 24 h. After 24 h, 100 µL of MTT in PBS was added to each well and incubated at 37 °C for 4h. The formazan crystals were formed and then solubilized in 100 µL of acidified isopropanol, and then the absorbance was measured at 630 nm by using a microplate reader (RADIM SEAC Sirio S, Pomezia, Italy). The percentages of cell inhibition were determined using the following formula:

$$\%\ \text{Cell inhibition} = (1 - \text{OD (absorbance) test/OD Control}) \times 100$$

This assay was carried out in triplicate. The IC_{50} value was determined from the % cell inhibition and concentration curve (Thakur et al., 2005).

For further anticancer mechanistic investigation, HepG2 cells were incubated with 20 and 40 µg/ml of bacterial extract for 24 h. Cisplatin (UNISTIN®, EIMC United Pharmaceuticals, Egypt, 3 µg/ml) was used as a positive control (Qu et al., 2004) and DMSO (8 µL/ml) as negative solvent control.

2.3.5 The bacterial extract cytotoxicity on normal human peripheral blood lymphocytes (HPBL):

2.3.5.1 HPBL culturing and safety evaluation:

The HPBL was extracted from three male volunteers (all healthy and non-smokers) to test the toxicity of the extract. Sterile syringes were used to draw peripheral venous blood samples, which were subsequently placed in sterile tubes with K2-EDTA (KEMICO vacutainer, Cairo, Egypt). The samples were prepared for culture in RPMI-1640 medium at 37 °C with a humidified 5% CO_2 incubator. The media was supplemented with 15% fetal calf serum, phytohemagglutinin (2%), and 1% (100 U/ml penicillin and 100 µg/ml streptomycin). Different concentrations of extract were applied after the cultures had been set up for 48 hours, and they were left for another 24 hours. The cultures were centrifuged for five minutes (Sigma, Osterode, Germany) at 1000 rpm to isolate HPBL. The pellets were then treated for fifteen minutes with five times the erythrocyte lysing solution (0.015 M NH4C1, 1 mM $NaHCO_3$, and 0.1 mM EDTA). Until a white pellet of lymphocytes formed, the incubation process was repeated (El-Garawani, 2015). All reagents were ordered from Lonza, Switzerland.

2.3.5.2 Acridine orange/ethidium bromide (AO/EB) dual fluorescent staining:

AO/EB staining was performed to investigate the viability of cells. Briefly, 4 µL of treated and control cell suspensions were labeled with 1 µl AO/EB solution (100 µg/ml AO and 100 µg/ml EB) on glass slides and examined immediately by a fluorescent microscope (Olympus BX41, Tokyo, Japan) at 400× magnification. Randomly, five fields were observed, and 200 cells were counted from each. Two types of cells were observed, based on the emitted fluorescence; viable cells were green-colored cells with intact structures, and late apoptotic or dead cells showed an orange-to-red color (Liu et al., 2015).

2.4 Statistical analysis:

The mean ± standard deviation (SD) is used to report the data. A one-way analysis of variance was used to examine the data for comparisons between several variables. The statistical tool SPSS version 17.1 software was utilized to compare the significant differences between groups utilizing Duncan's test as a post-hoc test. The significance level was set at $P < 0.05$.

3. Results

3.1 Biochemical characterization

A key biological trait of some bacteria that is widely acknowledged is motility, which serves as the foundation for classification in many taxonomic systems. To distinguish between species, motility is frequently seen as a crucial characteristic, and distinct species have occasionally been created based on the presence or absence of motility. The microscopic examination of the *Marinobacter HA2* strain suspension revealed that this strain was motile. A biochemical test for aerobic organisms is the catalase test, which allows us to observe the microbial synthesis of catalase enzymes. The Catalase enzyme is the most prevalent enzyme present in all living things, which catalyzes the breakdown of hydrogen peroxide to release oxygen and water. The results show that the *Marinobacter HA2* strain was able to produce catalase enzyme. The oxidase test was conducted to detect the presence of a cytochrome oxidase enzyme. In bacteria, the enzyme catalyzes the transport of electrons between electron donors and a redox dye called tetramethyl-p-phenylene-diamine. The purple color was seen,

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and the bacterial strain was positive for the oxidase test. The starch hydrolysis test was employed to identify the ability of the *Marinobacter HA2* strain to produce alpha amylase enzymes. The starch degradation was negative around bacterial strain growth. Urease is one of the oldest identified enzymes and provides survival to bacteria in inhospitable environments. The *Marinobacter HA2* strain was positive for urease production.

3.2 Anticancer activities:

3.2.1 Cytotoxicity on HepG2 cells:

The anticancer activity against the HepG2 cell line was assessed using an MTT assay. A promising cytotoxicity of the extract was given with 82±1.4 µg/ml maximal inhibitory concentration (IC50), Figure 1.

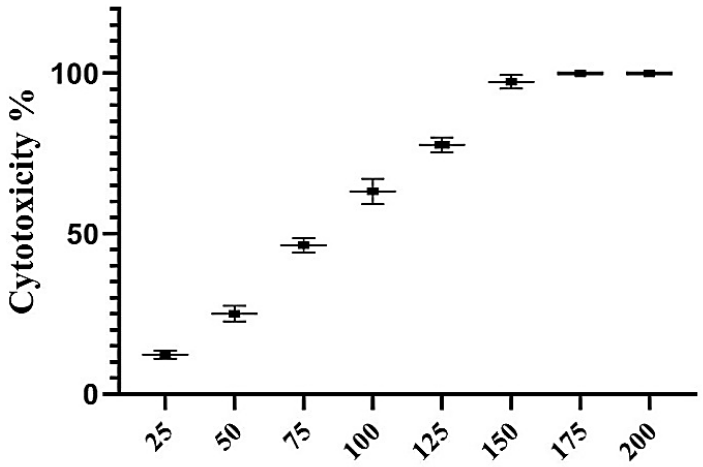

Figure 1. The cytotoxic effect of the *M. HA2* extract on HepG2 using MTT assay after 24 h. Incubation with serial concentrations (0-200 µg/ml) of the extract showed an IC_{50} of 82±1.4 µg/ml. Data are represented as (Mean ± SD) of three separate experiments (n = 3). *M. HA2: Marinobacter HA2.*

3.2.2 Extract toxicity on normal human peripheral lymphocytes:

3.2.2.1 Assessment of cytotoxicity on normal HPBL:

The AO/EB dual fluorescent staining was performed to assess the cytotoxicity on normal cells (HPBLs) after 24 hours of incubation with *M. HA2* crude extract. Results in dual fluorescent staining with AO/EB revealed lower percentages of apoptotic or necrotic cells as compared to high toxicity of MMC-treated cells. The records were 17.6±2.52 and 21.3±1.53 for 30 and 60 µg/ml, respectively Figure 2.

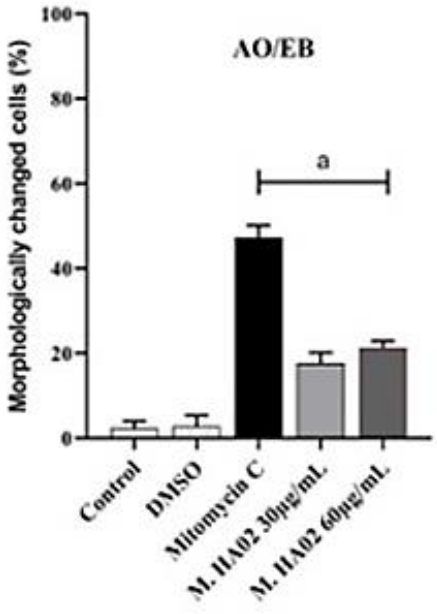

Figure 2. Effect of *M. HA2* on HPBL. Histograms represent the data (AO/EB) as Mean ± SD of three independent experiments (n=3). Bars, standard deviation and a: significant ($P < 0.05$) compared to the untreated cells. *M. HA2*: *Marinobacter HA2*; Mitomycin C (0.5 µg/ml).

4. Discussion:

Hypersaline ecosystems, which are widely distributed throughout the world, are dominated by halophilic microorganisms (Oren, 2008). Haloalkaliphilic microorganisms can survive and grow in unusual habitats under extreme salinity and pH conditions (Kulkarni et al., 2011)**.** As a result of their ability to adapt to high salinity, halophilic bacteria have attracted the attention of researchers (Corral et al., 2019). The biochemical characters of the collected strain (*Marinobacter HA2*) demonstrated that it was positive for catalase, oxidase, and urease enzyme production. Conversely, it could not hydrolyze starch, and additionally, its motility was observed under the light microscope. Bioactive compounds from microbial and marine origins are increasingly being used in pharmaceuticals and other biotechnological applications (Poli et al., 2009; Saeed et al., 2014). In our study, the biochemical characterization of newly identified *Marinobacter HA2* bacteria was tested. Moreover, the extracellular metabolite extract was tested for its anticancer properties against the hepatocellular carcinoma cell line. The results indicated that *Marinobacter HA2* crude extract had a significant effect on HepG2 cells. It was evident from the observed cytotoxic effect using MTT assay that apoptosis had been induced in the treated cells. Following previous studies using microbial extracts, these findings were also confirmed (Bitzer et al. 2006; Sagar et al. 2013). Moreover, even at higher concentrations, the extract is very unlikely to be cytotoxic or genotoxic to normal human lymphocytes.

In conclusion, the high level of bioactive compounds produced by microbial communities that thrive under extreme conditions captivate our attention to

produce valuable bioactive compounds. In this study, bacteria under extreme conditions of high salinity can produce secondary metabolites of anticancer effect.

Conflict of interest: NIL

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