

Anticancer and Apoptotic Effects of Ectoine and Hydroxyectoine on Non-Small Cell Lung Cancer cells: An in-vitro Investigation

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Abstract

Introduction: Streptomyces manage the osmotic stress by collecting compatible solutes or osmolytes. Ectoine, 1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid, and 5-hydroxyectoine are among the most common osmolytes of this bacteria and have an active role in protecting the genetic material of this microorganism as well as providing resistance to environmental pressures. In this research, the antiproliferative and apoptotic effects of ectoine and 5-hydroxyectoine on human non-small cell lung cancer (NSCLC) cell line (QU-DB) are investigated.

Methods: QU-DB cells were treated by five doses of ectoine and hydroxyectoine for 12, 24 and 48 hours, and the cytotoxic properties were evaluated by MTT assay. Then, the DAPI fluorescent staining was carried out for monitoring of the cells' nuclear morphology. Recognition and quantification of the apoptosis were done; using the Annexin-V-Fluos technique.

Results: It has been verified that both ectoine and hydroxyectoine persuaded apoptosis on QU-DB cells in a dose-dependently manner. Also, no cytotoxicity effects of these compatible solutes were observed on normal cells (HFLF-PI5).

Conclusions: Ectoine and hydroxyectoine are two natural metabolites which induce apoptosis in lung cancer cells. They don't have any toxic effect on normal cells and can be used as auxiliary drugs or even as a medicine for the treatment of lung cancer after further optimization of the formulations.

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INTRODUCTION

Lung cancer is the foremost wide-reaching cancer-related deaths that may be caused by cigarette

smoking, chemical exposure, radon gas, and air pollution [1-3]. Lung cancer does not usually display

any important symptoms before the metastasis stage. So, on time diagnosis would be the main issue for increasing the survival of patients with lung cancer [4]. Remedial methods which have been broadly used for the treatment of lung cancer include chemotherapy, radiotherapy, and surgery as well as several drugs such as cisplatin, etoposide, celecoxib, and paclitaxel [5-9]. With all this, there are several disadvantages using these techniques. For example, chemotherapy has definite side effects such as severe toxicity [10]. Recent studies have shown that herbal drugs are attractive manners for medical treatment of lung cancer with few side effects. The killing of the cancerous cell via the programmed cell death pathway; known as apoptosis, is the best goal of anticancer agent's usage with an insignificant side effect on normal cells. Various studies have shown that people with cancer usually have an interest in using plants or herbal products to achieve health and recovery [11]. Moreover, numerous natural compounds and metabolites of plants, fungi, and bacteria are useful and effective in sensitizing the conventional agents [12].

Various microorganisms store a particular class of biological osmolytes, the compatible solutes, to tolerate high osmolarity of ecological niches and prevent cellular hydration and turgor [13-15]. Along with their mentioned applications as osmoprotectants, compatible solutes are also protein-stabilizer to maintain the proper folding of polypeptides both in vitro and in vivo under denaturing conditions [16-19]. So, according to the documentation, they are mentioned as chemical chaperones, and as protectants against heat and cold stress [16, 20-23]

The ectoine [(S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid], tetrahydropyrimidines ectoine, and its hydroxylated derivative 5-hydroxyectoine [(4S,5S)-5-hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] are among the most commonly bacterially used compatible solutes [23].

They also are the most frequent osmolytes which are usually found in *Streptomyces* [24]. Some studies presented promising results about the preventive effect of ectoine on the induction of anti-inflammation and anti-apoptotic signaling in the immune system [24].

Although ectoine has been introduced as a defensive agent in animals and humans [25], there are limited

documents to prove their therapeutical potentials.

As described, in the present research, the goal was to investigate the antiproliferation effects of ectoine and hydroxyectoine in QU-DB cells as an in vitro model of non-small cell lung cancer (NSCLC).

This research was designed to provide future therapeutic strategies for clinical management of lung cancer.

METHODS

Chemotherapeutic Agent and Cell Culture

Ectoine and hydroxyectoine were attained from the BIOTREND chemical AG (Zurich, Switzerland). Concentrations of 1 to 20 $\mu\text{mol/L}$ were prepared from each of them. Human lung carcinoma cell line (QU-DB) was prepared from the National Cell Bank of Iran (NCBI) and was cultured in Dulbecco's Modified Eagle's medium (DMEM); complemented with 10% fetal bovine serum in a moistened atmosphere of 5% CO_2 and 95% air at 37°C. Human lung cell line (HFLF-PI5) was used as the control group (Data not showed). HFLF-PI5 was cultured in RPMI in the same conditions.

Cell Viability

QU-DB cells were treated in triplicate for each test with different concentrations (1-20 micromolar) of ectoine and hydroxyectoine. To determine the effect of this compatible solutes on cell proliferation, the MTT assay was done after the treatment. Accordingly, 10 μl of MTT reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, was added to each well. Following another incubation period of 4 hours at 37°C, the supernatant was removed from each well. The colored formazan crystals, which were produced from MTT, were dissolved in Dimethyl sulfoxide (DMSO; sigma. USA) and absorbance was then measured at 570 nm by an ELISA plate reader (ELX800TM, USA).

DAPI Staining

The blue-fluorescent DAPI was used for the morphological detection of ectoine and hydroxyectoine-induced apoptotic cells according to the standard protocol [12].

Flow Cytometry

For the recognition and quantification of apoptosis and its distinction from necrosis, the Annexin-V-Fluos staining method was used. Accordingly,

20 µl of Annexin-V-Fluos labeling reagent was pre-diluted in 1ml incubation buffer and added to 20 µl of propidium iodide (PI) solution. The cell pellets which were treated by ectoine and hydroxyectoine were resuspended in 100 µl of Annexin-V-Fluos solution after incubation for 15 min at 25°C. The evaluation was done on a flow cytometer (BD, USA) and data analysis was done by a Flowmax software [26, 27].

RESULTS

MTT Assay

As shown in Figure 1 and Figure 2, the repressed proliferation of these cancer cell lines were significantly different in concentrations ranging from 1 to 2 µmol/L and during the 24 hours of incubation.

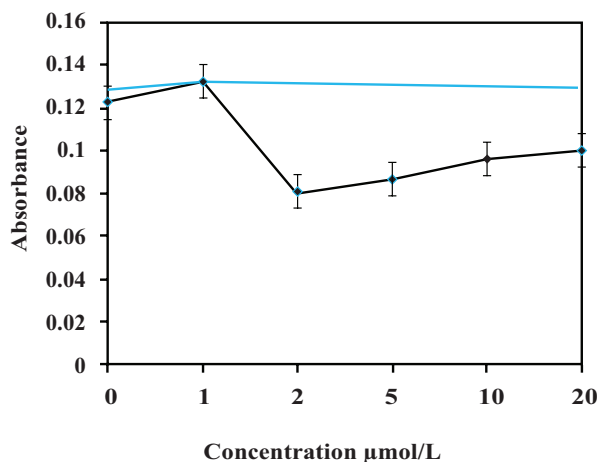


Figure 1: The Absorbance of QU-DB Cells at 570 nm After 24 Hours of Treatment With Different Concentrations of Ectoine
The blue line indicates the absorbance of normal cells in a similar condition.

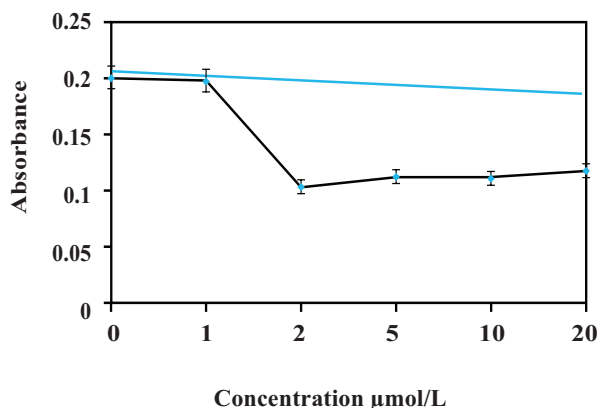


Figure 2: The Absorbance of QU-DB Cells at 570 nm After 24 Hours of Treatment With Different Concentrations of Hydroxyectoine
The blue line indicates the absorbance of normal cells in a similar condition.

The results showed that the minimum percentage of cell viability after treatment with various concentrations of ectoine was 77% being obtained at the concentration of 2 µmol/L. After treatment with different concentrations of hydroxyectoine, the minimum percentage of cell viability was 67.7% at the same concentration.

DAPI Staining

As displayed in Figure 3, the extreme rise in the number of apoptotic cells were found in the same concentration of 2mM for both ectoine and hydroxyectoine.

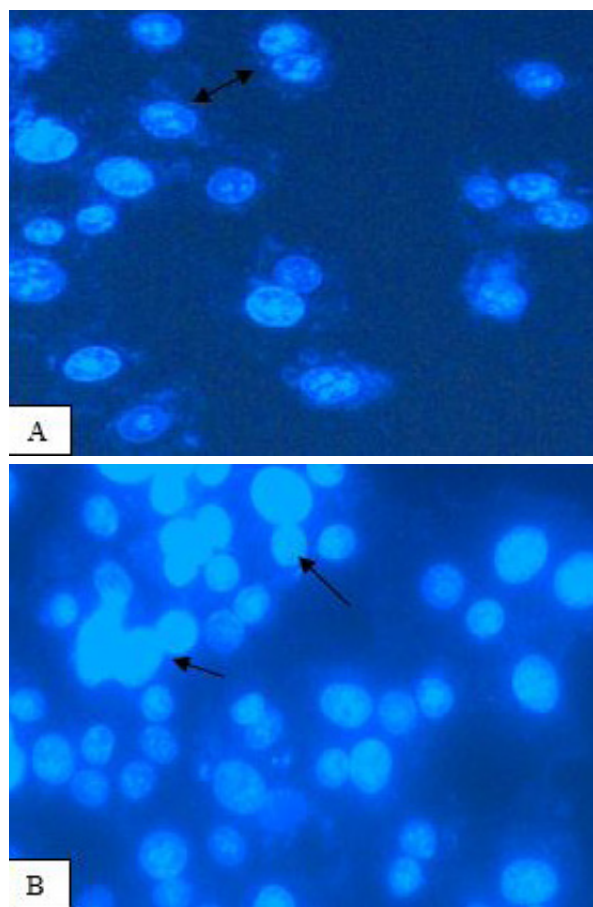


Figure 3: A) DAPI Staining of QU-DB Cells Affected by Ectoine Treatment for 24 Hours; B) DAPI Staining of QU-DB Cells Treated With Hydroxyectoine for 24 Hours
→ indicates normal cells, ↔ indicates apoptotic cells

Flow Cytometry Analysis of Apoptotic Cancer Cells
The Flow Cytometric analysis evidently discriminates standard (living) cells with low Annexin and low PI staining, apoptotic cells with high Annexin and low PI staining, and necrotic cells with high Annexin and high PI staining. As represented in Figure 4, the maximum apoptosis induction was observed in the concentration of 2 mM for both ectoine (20.97%) and hydroxyectoine (20.97%).

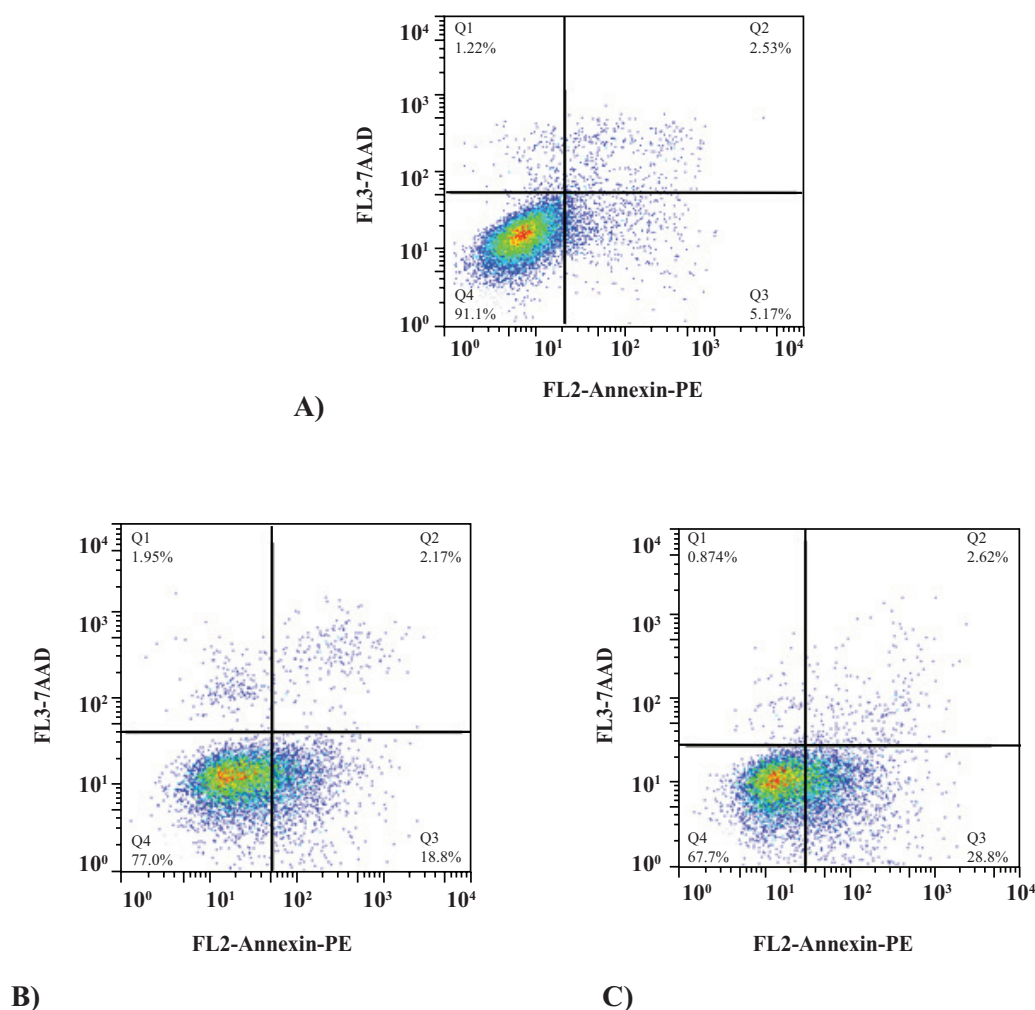


Figure 4: Flow Cytometry Analysis of Apoptotic QU-DB Cells
A) Control; B) Cells Treated by Ectoine; C) Cells Treated by Hydroxyectoine.

DISCUSSION

The metabolites that are secreted by some microorganisms for their survival and protection, may have beneficial effects for other organisms. For example, *Streptomyces* produce and accumulate compatible solutes such as ectoine and hydroxyectoine for environmental stresses protection. The widespread expansion of natural metabolites such as ectoines with biologic activity and therapeutic potentials has increased the attention of scientists. Due to the significant increase in demand for these new therapeutic agents, efforts have been made to optimize the production processes of such metabolites and allow their applications to protect macromolecules, cells, and tissues [28]. Considering the fact that compatible solutes have minor toxic potentials, this group of substances may be used in clinical trials to evaluate their efficacy in the prevention and management of lung cancer as

a substitute for old-style methods. Today, according to the new approaches in the field of biomimetics in drug delivery and treatment, these natural metabolites can be used with excellent efficiency, but without noticeable side effects [29, 30]. Also, they can be used in the design and manufacture of new anticancer nano-bio hybrid drugs [31]. It was demonstrated that ectoine and hydroxyectoine, dose-dependently reduced the viability of QU-DB cell line, the human lung cancer cell. Briefly, both ectoine and hydroxyectoine have cytotoxic effects on these cancer cells at 2mM of concentration. In addition, hydroxyectoine has more cytotoxic effects on QU-DB cells than ectoine.

Previous studies have shown that ectoine activates the MAPK A ERK1/2, intracellular signaling pathways, and cell-cell interaction [32]. Although ectoine is used as an anti-inflammatory agent in pharmaceutical sprays, the apoptotic study

on lung cancer cells was conducted for the first time. Biological, genetical, and in vivo studies will allow a deeper understanding of ectoine and hydroxyectoine roles in cell signaling pathways. Bioprocess and metabolic engineering strategies are needed prior to the clinical applications of ectoines. So, further studies such as cell signaling and in vivo investigations are under process; using controlled systems like bioreactors for process optimization. Based on the new findings of this in vitro research, we demonstrated for the first time that both ectoine and hydroxyectoine inhibited the rapid and irregular growth of lung cancer cells. However, further studies are required in animal models to find the genetic pathways involved in apoptosis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS APPROVAL

Not applicable.

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