Evaluation Effect of Eucalyptus Sargentii and Doxorubicin on A549 Cell Line in Lung Cancer

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ABSTRACT

Eucalyptus sargentii belongs to the Myrtaceae family of Australian trees. One of the species of Eucalyptus has cultivated in Iran. Eucalyptus extract is a promising natural source of anti-cancer molecules. In this study, Growth inhibitory effects of Eucalyptus extract and doxorubicin were assessed by MTT assay. The effect of Eucalyptus extract and doxorubicin on apoptosis in the lung adenocarcinoma A549 cell line was investigated by flow cytometry. Cell cycle analysis was evaluated by flow cytometry. Level of Gene expression of Bax, Bcl-2 and KRAS were determined by Real time PCR. Our data showed that Doxorubicin (1.5 \( \mu \)g ml\(^{-1}\)), Eucalyptus (20 \( \mu \)g ml\(^{-1}\)) and their combination inhibited proliferation and induced apoptosis in A549. Level of mRNA expression of Bax was increased while expression of Bcl-2 and KRAS were decreased at 48 h. During flowcytometry (PI) test revealed that the highest apoptosis occurred at a concentration of combination of Doxorubicin and Eucalyptus. Thruh the cell cycle, the highest percentage of cells in the G0/G1 phase was observed in the treatment with combination of Doxorubicin and Eucalyptus. According to the results combination of Doxorubicin and Eucalyptus extract showed higher level apoptosis in comparison with agents separately. Furthermore, increase of BAX mRNA expression indicated pro-apoptotic function.

Keywords: A549 lung adenocarcinoma cell line, Bax, Bcl-2, Doxorubicin, Eucalyptus, KRAS

INTRODUCTION

The Myrtaceae family contains many trees. This family can be found in in Australia, tropical America, Africa and Asia [1]. Eucalyptus is the most important genera of Myrtaceae [2]. Although most plants are native to Australia and Tasmanis [3], they have been cultivated in many countries, including Iran. In folk medicine, Eucalyptus leaves have wounds healing, antibacterial and antifungal activities [4-9]. Extracts of several Eucalyptus species have been shown to possess cytotoxic and antitumor activities [10-11]. Cancer is a major cause of death and chemotherapy is an important treatment for cancers. However most of drug used in cancer chemotherapy have side effects on non-tumor cells. Thus natural drugs, which are less toxic with fewer side effect are important. Lung cancer classifies into two subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [12-13]. NSCLC represents 85% of cases of lung cancer. RAS proteins, including KRAS, are intracellular guanine nucleotide binding proteins, which belong to the family of GTPases. The exchange of GDP-GTP is regulated by Guanine nucleotide exchange factors (GEFs) decrease the affinity of RAS proteins for GDP and favor GTP binding that results in RAS activation, while GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity to regulate the RAS cycle [14-17]. Most mutations in RAS genes affect exons 2 and 3. These mutations impair the GTPase activity promoting the active GTP-bound state. In some cancers, RAS are mutated and GTPase activity is impaired. So this pathway has been activated whole the time that induces cancers [18-20]. Up to now 9 point mutations have been identified in KRAS, which accounts for 95% of cancers [21-22]. Four signaling pathways after activation of RAS are: 1- RAS/RAF/MEK/ERK plays a role in controlling cell proliferation by regulating cellular regimens and are often overactive in cancers. 2- RAS/PI3K/PDK/AKT/MTOR, this signaling pathway has the role of cell survival. 3- RAL GDS/RAL/PLD role in controlling the accumulation of...
Vesicles. 4- RAS/ IAM1 that play a role in the organization of a cellular skeleton [23]. Apoptosis or planned cell death is a natural way to eliminate old cells from the body. Many anticancer therapies induce apoptosis and related cell death networks to kill malignant cells [24-26]. The Bcl-2 family proteins are involved in positive and negative regulation of apoptotic cell death [27]. Among the anti-apoptotic members, Bcl-2 and Bcl-XL are negative regulators of cell death, preventing cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types, whereas others, such as Bax and Bid promote or accelerate cell death [24]. Doxorubicin, an anthracycline antibiotic, is used in lung, breast, ovarian, thyroid and gastric cancers [28]. The current study emphasizes on the evaluation of biological activities of extract of Eucalyptus sargentii leaves and doxorubicin on A549 lung cancer.

MATERIAL AND METHODS

Reagents

Doxorubicin was purchased from Pfizer (Australia). Eucalyptus sargentii was purchased from Iranian biological research center. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Atocel (Budapest). DNA marker was purchased from Fermentas (USA). DMSO, DEPC, annexin V-propidium iodide (PI) apoptosis detection kit were obtained from Sigma. Fetal calf serum (FCS) and RPMI1640 medium were purchased from Biosera (UK). RPMI-1640 supplemented with 3.7 g l⁻¹ NaHCO₃, 30 mg l⁻¹ asparagine, 100 U ml⁻¹ penicillin, and 10 mg ml⁻¹ streptomycin (Biosera) pH 7.2 was prepared and after sterilization by 0.2-µm Millipore filter kept at 4 °C before use. Human lung cancer cell line A549 was obtained from the Pasteur Institute of Iran. The cells were cultured in RPMI-1640 medium by incubation in humidified condition with 5% CO₂ at 37 °C.

Preparation of Ethanolic Extract of Eucalyptus

1000 ml of 70% ethanol was added to 200 g of milled Eucalyptus and mixed thoroughly by glass stirrer an hour and after 24 h, the mixture was smooth using a funnel and 4 layers of sterile gauze. To remove impurities in the extract, centrifugation at rpm2500 for 20 min at 4 °C was performed. Complete withdrawal of solvent was carried out using a distiller. The extract was sterilized via 0.45 µm sterile syringe filter and divided into micro tubes and stored at -80 °C. After adding of RPMI medium into dried extract, 1, 5, 10, 20, 50, 100, 150 and 200 µg ml⁻¹ concentrations were prepared [29].

Cytotoxicity Assay

A549 (1 × 10⁵ cells/ml) were cultured in the absence and presence of various concentrations of doxorubicin (0-5 µg ml⁻¹), Eucalyptus (0-200 µg ml⁻¹) and their combination for 24 and 48 h at 37 °C and 5% CO₂. The proliferation rate of cells was analyzed by MTT assay [30]. The cells (10⁴ cells/well) were cultured in RPMI in 96-well cell culture plates and treated, then, 10 µl of MTTC (5 mg ml⁻¹ in H₂O) was added to each well and the cells were incubated for 4 h in humidified condition and 5% CO₂ at 37 °C. The medium was removed, 150 µl dimethyl sulfoxide (DMSO) was added to each well to solubilize the resulting formazan crystals, and the absorbance monitored at 570 nm by Awareness ELIZA reader. Cell viability was estimated as the percentage of the control.

RNA Extraction and Real-time PCR

Total RNA was extracted from the doxorubicin, Eucalyptus treated cells and the controls using RNX-Plus solution (Bioflux) following the manufacturer’s instruction. The RNA (2 µg) was reversed transcribed into complementary DNA (cDNA) using two-step reverse transcription polymerase chain reaction (RT-PCR) Kit (Thermo scientific) according to the manufacturer’s protocol. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in final volume of 20 µl using quantitative polymerase chain reaction (qPCR) probe Master (Amplicon, UK). PCR profile was 95 °C for 3 min as pre-denaturation step, 40 amplification cycles at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 15 s. Final extension was carried out at 72 °C for 10 min. Gene-specific PCR primers were as follows: the Bax forward: 5′-GAGCTGCAGAGGATGATTGC, Bax reverse: 5′-AATTGGGAAGTTTCAAATCAGC, Bcl-2 forward: 5′-ATTGGGAAGTTTCAAATCAGC, Bcl-2 reverse: 5′-GAGCTGCAGAGGATGATTGC, KRAS forward: 5′-TGAGGACTGGGAGGGCTTT-3′,
KRAS reverse: 5’-ACCATAAGTGACATCTTCAGAG-3’, β-Actin forward: 5’-TCCTCCTGAGCGCAAGTC-3’, β-Actin reverse: CGGTGGACGATGGAGGGCC-3’ (Bioneer, korea). All gene sequences were acquired from the NCBI website and Primer3, and oligo analyzer and gene runner programs were used to design primers for the specific genes and their specificity analyzed by the NCBI Blast Program. β-Actin was used as an endogenous control and quantitation of gene expression determined using ΔΔCt calculation, where Ct is the threshold cycle. BAX, BcL-2, KRAS and β-actin mRNA expression levels were then analyzed as fold-change by the 2−ΔΔCt [31]. The fold change profile was performed by a Bioneer Exicycler™ 96 and data were analyzed using REST 2009.

Apoptosis Assay

The control and drug-treated cells (3 × 10^5) were stained by annexin V/PI according to the manufacturer’s protocol for 20 min in the dark at room temperature. Flow cytometry analysis was performed using BD FACS Calibur Flow Cytometry System (Becton Dickinson, USA), and the percentage of apoptotic cells was determined by BDPAC software. For cell cycle analysis, the cells (3 × 10^5) were cultured in the absence and presence of doxorubicin and extraction of Eucalyptus fixed with 70% ethanol at 4 °C for 2 h, and stained in PBS containing 20 µg ml⁻¹ PI and 20 µg ml⁻¹ RNase at 37 °C for 30 min in the dark. The cell cycle profile was performed by a BD FACS Calibur Flow Cytometry, and data were analyzed using BDPAC software.

Statistical Analysis

All in vitro experiments were performed in triplicate, and results have been expressed as the mean ± standard deviation (SE), Student’s t test and one-way analysis.

RESULTS

Toxicity Effect of Eucalyptus on A549 Cells

The cell proliferation of A549 which treated with indicated concentration of doxorubicin (0-5 µg ml⁻¹) and Eucalyptus (0-200 µg ml⁻¹) were assessed by MTT assay for 24 h and 48 h. We perceived both time- and dose-dependent effect of compounds. We evaluated effect of selected dose of doxorubicin (1.5 µg ml⁻¹) and Eucalyptus (20 µg ml⁻¹) (Fig. 1). Data indicated that doxorubicin and Eucalyptus separately and their combination significantly decrease cell proliferation in comparison to the control. In the absence of the drug, the cells represent about 100% viability but in the presence of doxorubicin, viability is decreased to 51% at 1.5 µg ml⁻¹. Our data demonstrated that viability is decreased 54% at 20 µg ml⁻¹ of Eucalyptus. When the cells were cultured for longer time (48 h), viability of viable cells are decreased to 37% at 1.5 µg ml⁻¹ of doxorubicin and 49% at 20 µg ml⁻¹ of Eucalyptus.

The Effect of Doxorubicin and Eucalyptus on BAX and BcL-2 Expression

To evaluate the expression of Bax and BcL-2 mRNA, total RNA was extracted from A 549 cells cultured with selective concentrations of doxorubicin (1.5 µg ml⁻¹), Eucalyptus (20 µg ml⁻¹) and also their combination for 24 and 48 h. Then we converted to cDNA and analysis of gene expression was performed by qPCR. β-actin was used to establish normalized expression (ΔΔCt). Data indicated that the mRNA expression of Bax was up regulated when A549 cells were treated with doxorubicin and Eucalyptus alone and their combination. The significant up regulation of Bax mRNA expression was observed when cells were treated by 1.5 µg ml⁻¹ of doxorubicin and 20 µg ml⁻¹ of Eucalyptus (Fig. 2). In addition, our results showed that doxorubicin and also in combination with Eucalyptus led to down regulation of mRNA expression of BcL-2. KRAS is one of the most important proteins in metastasis. Expression of this protein was also evaluated in the identical condition as used for Bax and BcL-2. The result in Fig. 2 shows that, in contrast to Bax, KRAS mRNA expression is down-regulated. At 1.5 µg ml⁻¹ concentrations of the doxorubicin the level of KRAS expression was decreased about 70% of the control. Our data indicated that the combination (doxorubicin 1.5 µg ml⁻¹, Eucalyptus 20 µg ml⁻¹) significantly down regulated the mRNA expression of KRAS.

Eucalyptus Induces Apoptosis in A 549 Cells

For this purpose, the control and drug-treated cells were stained by annexin V/PI and analyzed by flow cytometry. In this procedure, viable cells are annexin V- and PI-, early apoptotic cells are annexin V+ and PI-, and necrotic cells are annexin V+ and PI+. As is seen in Fig. 3, in the absence
Fig. 1. Cell Proliferation in A549. Effects of Doxorubicin and Eucalyptus on cell proliferation of A549 cell lines. The antiproliferative effects of Doxorubicin (0-5 µg ml⁻¹), Eucalyptus (0-200 µg ml⁻¹) and their combinations on A549 were assessed by MTT assay after 24 h and 48 h.
of the doxorubicin and Eucalyptus, the maximum cells were viable and the amount of apoptotic and necrotic cells were little. Upon addition of the doxorubicin, a shift occurs from live cells (lower-left quadrant) to early apoptotic cells (lower-right quadrant). In addition, percent of apoptotic cells is increased to 26.02%, 28.27% and 46.33% after exposure to Eucalyptus 20 µg ml⁻¹, doxorubicin 1.5 µg ml⁻¹, Eucalyptus 20 µg ml⁻¹ and doxorubicin 1.5 µg ml⁻¹, whereas necrosis was negligible and did not exceed 12%. DNA content of A549 cells evaluated during cell cycle. In present study after treatment of A549 the cell population in sub G1 phase increased in all doses especially when cells

Fig. 2. The effect of Doxorubicin and Eucalyptus on the expression levels of BAX, Bcl-2 and KRAS was determined by qRT-PCR analysis in A549 cells. A: effect of Doxo/Euca on Bax gene expression at 24 h and 48 h. B: effect of Doxo/Euca on Bcl-2 gene expression at 24 h and 48 h. C: effect of Doxo Euca on KRAS gene expression at 24 h and 48 h.
treated with combination of doxorubicin and *Eucalyptus* for 48h. Our data indicated that combination increased the hypodiploid sub G1 DNA fraction in dose dependet manner (0 to 32.99%) indicating apoptotic population (Fig. 4).

**DISCUSSIONS**

Lung cancer includes two subgroups: non-small cell lung cancer (NSCLC) and SCLC small cell lung cancer [32, 33]. The A549 NSCLC cell line was established in 1976 and widely studied for the study of lung cessation. A number of genetic alterations have been described in NSCLC, being Kristen Rat Sarcoma viral oncogene (*KRAS*), Epidermal Growth Factor Receptor (EGFR) and Anaplastic Lymphoma Kinase (ALK) the most commonly altered oncogenes acting as tumor genomic drivers [34]. Proteins of the Bcl-2 family dimerize,[35] although all possible heterodimers are not formed in the cells [36]. A widely accepted model postulates that homodimers of Bax promote apoptosis [27,37,38], and that the functional effect of Bcl-2 related proteins is to form competing heterodimers with Bax which are unable to promote apoptosis [39,40]. However, it seems that Bcl-2 and BAX are both able to regulate apoptosis independently [41,42]. In the present study, we investigated the effects of doxorubicin and extract of *Eucalyptus sargentii* leaves on expression of Bax, Bcl2 and *KRAS* in A549 lung cancer.

The results demonstrate that extract of *Eucalyptus sargentii* leaves has toxicity on A549 cells. Farhadul Islam and colleagues used *Eucalyptus* (25 mg kg⁻¹, 50, 100).
Eucalyptus had 96% antitumor effect on mice with EAC (a type of carcinoma). They reported that Eucalyptus had a significant effect on tumor reduction [43]. Farhadul Islam and his colleagues used methanol extracts of Eucalyptus (ME) on EAC. ME with doses (25 mg kg\(^{-1}\), 50, 100). ME in 100 mg kg\(^{-1}\) could inhibit the growth of cancer cells up to 96% [43]. P.M. Doll-Boscardin et al., examined the effect of Eucalyptus benthami on Jurkat cells. Eucalyptus concentrations were (3, 10, 30, 100 and 300 μg ml\(^{-1}\)). Eucalyptus benthami on Jurkat cells had IC50 = 56.51 μg ml\(^{-1}\), also IC50 value of Fruits of Eucalyptus globulus alone in A549 is about 10 μg ml\(^{-1}\) [44]. Doxorubicin, an anthracycline antibiotic, is widely used and known for its anticancer activity towards lung, breast, ovarian, thyroid and gastric cancers. Doxorubicin has toxicity on Non-small cell lung cancer (NSCLC) such as H460, H1299 and H23 [45-46]. Punnia et al indicated that in A549 cells, IC50 values of doxorubicin at 48 h was 17.83 nM [46].

KRAS expression in the cells that exposed to Leaves of Eucalyptus extraction alone and combination with
doxorubicin down-regulated compared to the control. In previous study researchers reported that KRAS gene mutations in many types of human cancers observed [47], such as pancreatic carcinomas (>80%), colon carcinomas (40-50%), and lung carcinomas (30-50%), and also in biliary tract malignancies, endometrial cancer, cervical cancer, bladder cancer, liver cancer, myeloid leukemia [48, 49] and breast cancer [50].

Bax expression in the cells exposed to Leaves of Eucalyptus extraction alone and combination with doxorubicin is up regulated compared to the control whereas Bcl-2 down regulated. The Bcl-2 family of proteins includes pro and anti-apoptotic activity. Bax pro apoptotic and Bcl-2 is anti-apoptotic agents. T.A. Pham et al., isolated a new fluoroquinol derivative from Eucalyptus globulus fruits that called eucalyptin B. eucalyptin B was studied for their apoptotic agents. Takahashi, R. Kokubo, M. Sakaino, Lett. Appl. Microbial. 39 (2004) 60.

Gene expression of HDAC8 decreased in A549 cells when treated with silver nanoparticles after treatment with Eucalyptus extract [53].

In conclusion, doxorubicin as a Bcl-2 inhibitor in combination with extract of Eucalyptus sargentii leaves have synergistic impact on the inhibition of cell proliferation and promotion of apoptosis in A549 lung cancer. Leaves of Eucalyptus sargentii extraction enhances the pro-apoptotic activity of doxorubicin in A549 lung cancer.

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