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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 µg ml^{-1}), *Eucalyptus* (20 µg 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 occured at a concentration of combination of Doxorubicin and *Eucalyptus*. Thrugh the cell cycle, the highest percentage of cells in the G_0/G_1 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 Tasmania [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

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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-dimethylthiazal2-yl)-2,5-diphenyl-tetrazolium 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} $NaHCO_3$, 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% CO_2 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 (1×10^4 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 ºC and 5% CO_2. The proliferation rate of cells was analyzed by MTT assay [30]. The cells (10^4 cells/well) were cultured in RPMI in 96-well cell culture plates and treated, then, 10 µl of MTT (5 mg ml^{-1} in H_2O) was added to each well and the cells were incubated for 4 h in humidified condition and 5% CO_2 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-3′, *Bax* reverse: 5′-AAGTTGCCGTCAGAAAACATG-3′, *Bcl-2* forward: 5′-ATTGGGAAGTTTCAAATCAGC-3′, *Bcl-2* reverse: 5′-CAGTCTACTTCCTCTGTGATGTTG-3′, *KRAS* forward: 5′-TGAGGACTGGGGAGGGCTTT-3′,

KRAS reverse: 5´-ACCATAGGTACATCTTCAGAG-3´, β-Actin forward: 5'-TCCTCCTGAGCGCAAGTAC-3', β-Actin reverse: CGGTGGACGATGGAGGGGCC-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 $2^{-\Delta\Delta Ct}$ [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

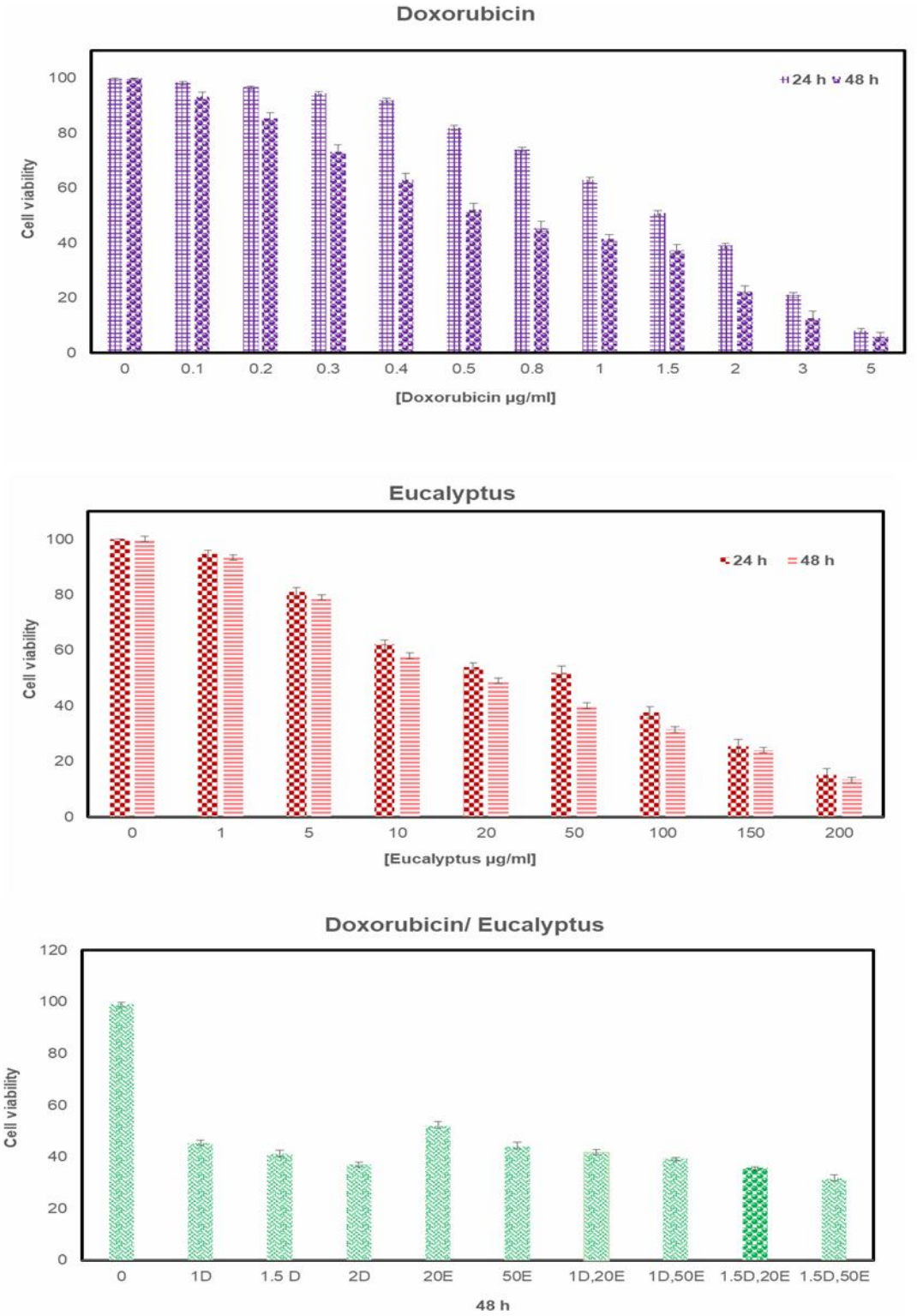

Fig. 1. Cell Proliferation in A549. Effects of Doxorubicin and *Eucalyptus* on cell proliferation of A549 cell lines. The antiproliferative effects of Doxorubicin (0-5 µg ml^{-1}), *Eucalyptus* (0-200 µg ml^{-1}) and their combinations on A549 were assessed by MTT assay after 24 h and 48 h.

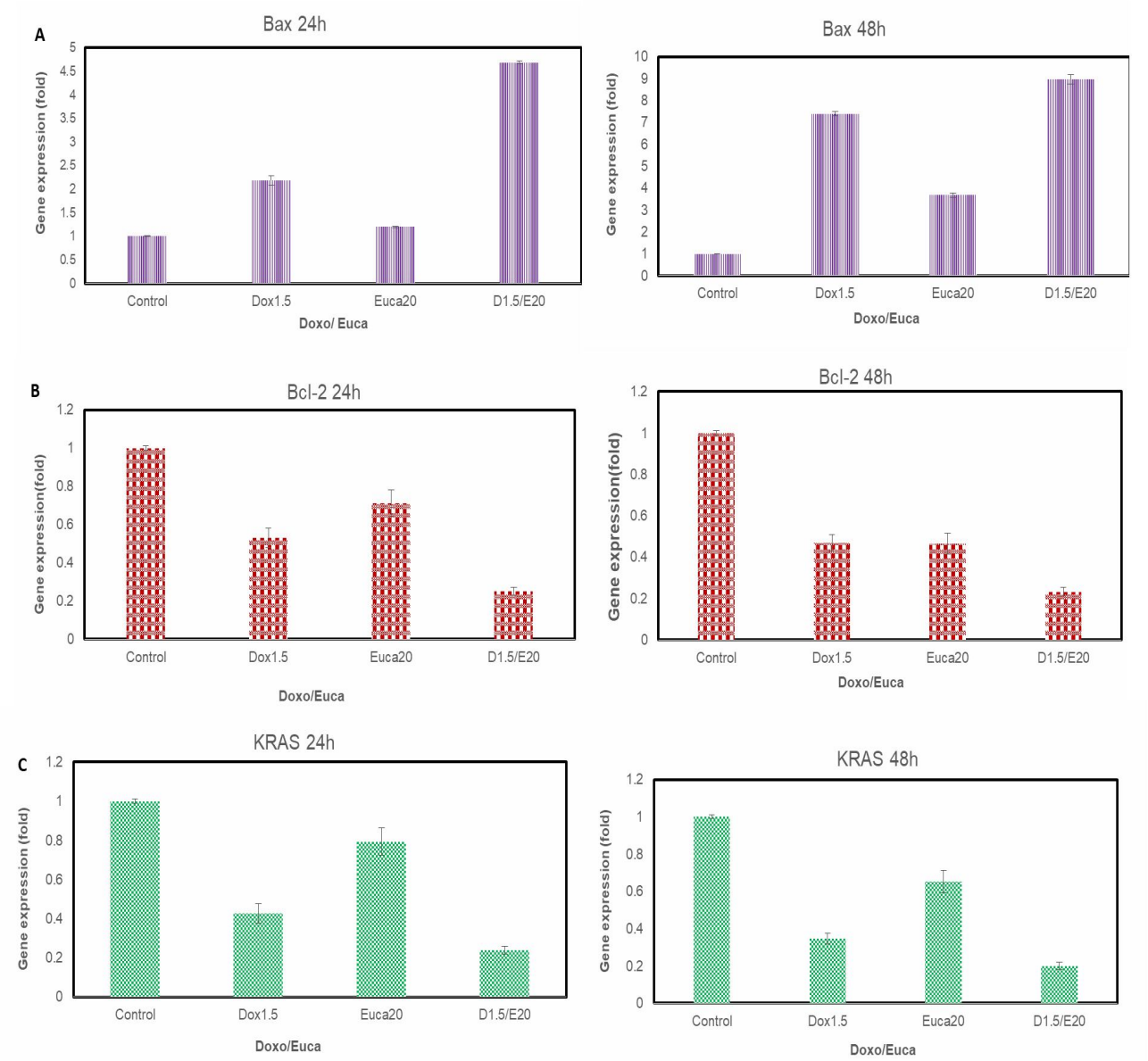

Fig. 2. The effect of Doxorubicin and *Eucalyptus* on the expression levels of *BAX*, *Bcl-2* and *KRAS* was determined by qRT-PCR analysis in A549 cells. A: effect of Doxo/Euca on *Bax* gene expression at 24 h and 48 h. B: effect of Doxo/Euca on *Bcl-2* gene expression at 24 h and 48 h. C: effect of Doxo Euca on *KRAS* gene expression at 24 h and 48 h.

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

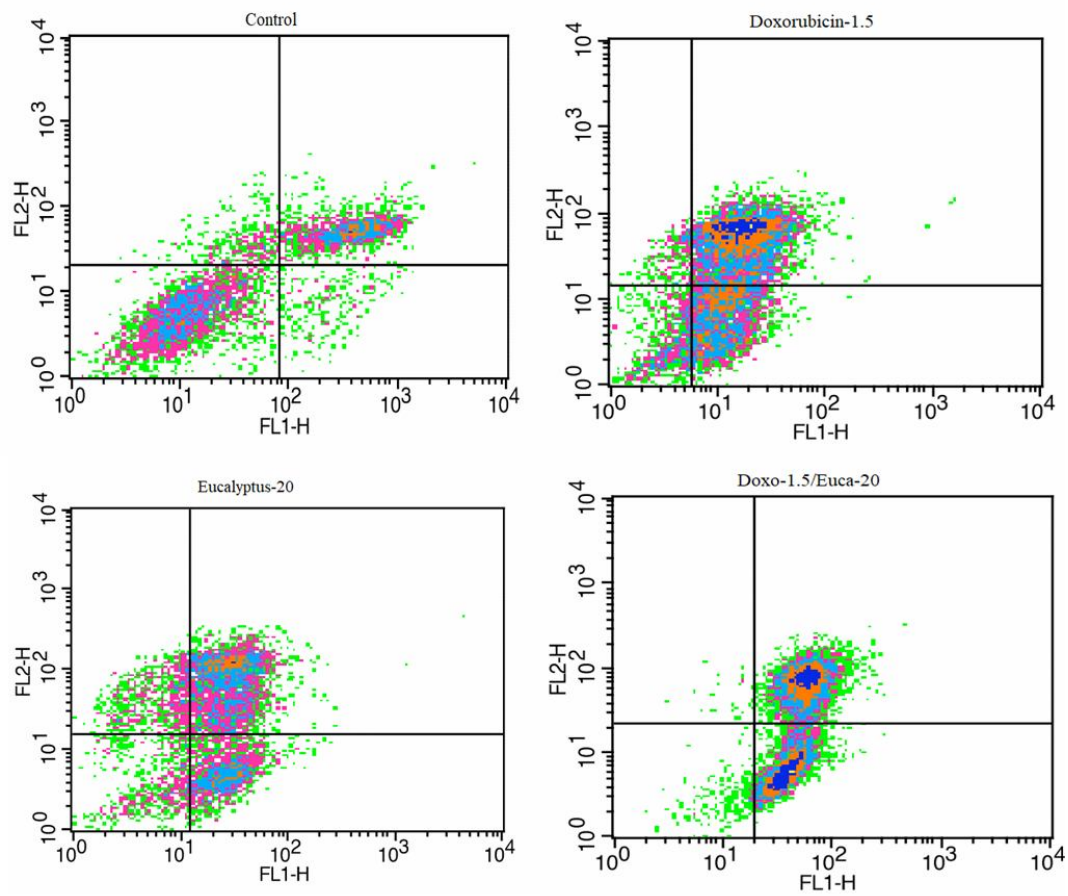

Fig. 3. Flow cytometry of A 549 cells treated with Doxorubicin (1.5 µg ml^{-1}) and *Eucalyptus* (20 µg ml^{-1}) and their combination. The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells.

treated with combination of doxorubicin and *Eucalyptus* for 48h. Our data indicated that combination increased the hypo diploid 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).

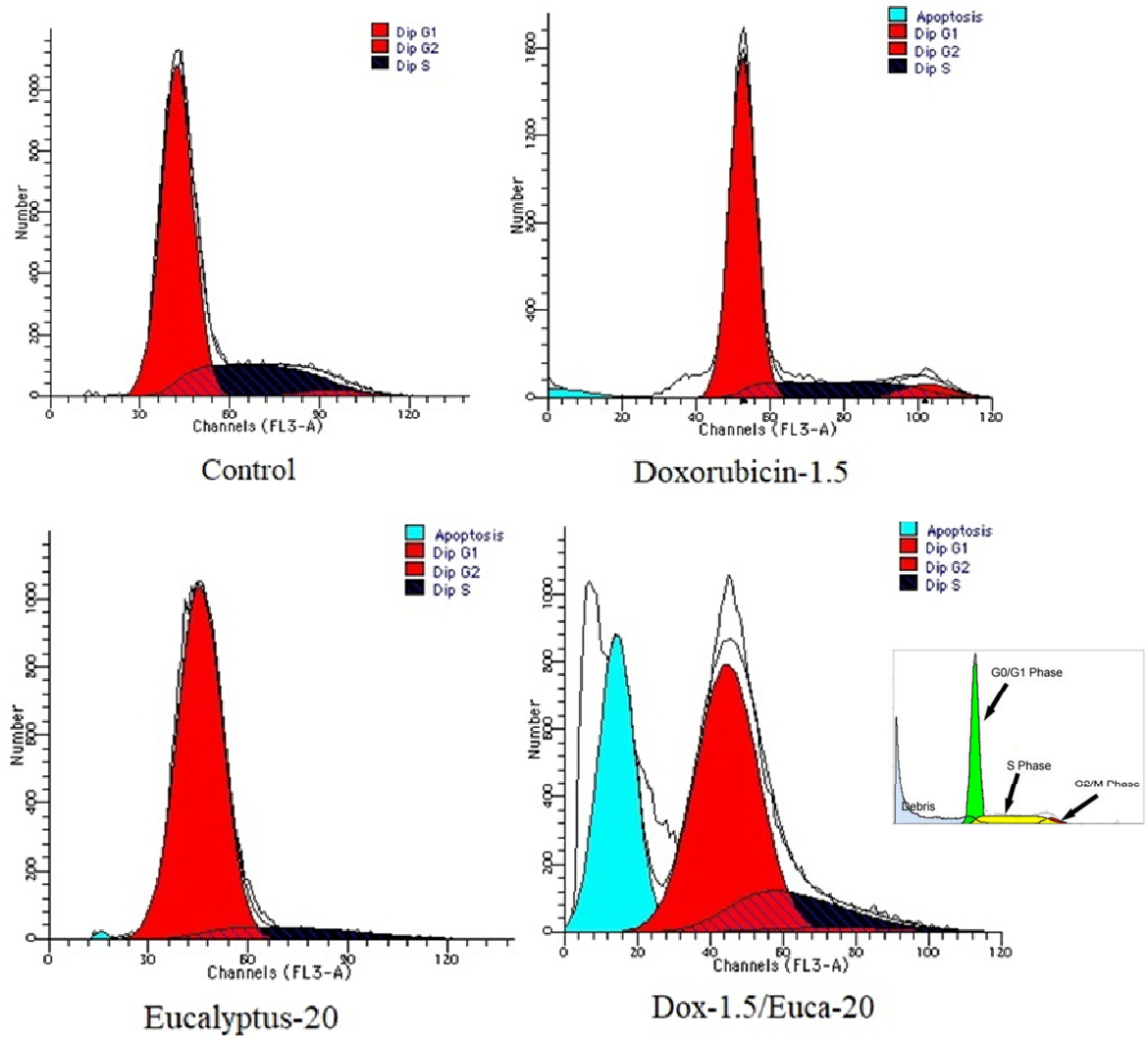

Fig. 4. Cell cycle flow cytometry analysis of A549. 48 h exposure to different concentrations of Doxorubicin and *Eucalyptus* reduces the number of cells at G2 phase and increases the amount of cells at G1 phase of the cell cycle in A549 cells and was observed significant accumulation of cells in theG0/G1 phase.

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 cytotoxic activity against lung cancer cells (A549). They observed that increasing apoptosis on A549 cancer cells [51]. This results are consistent with the results of this article. Sharifi *et al*. demonstrated that Doxorubicin decreased the anti-apoptotic Bcl-xL and increased pro-apoptotic Bax mRNA levels [52].

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 combinationwith 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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REFERENCES

[1] P.G. Wilson, M.M. O'Brien, P.A. Gadek, C.J .Quinn, Am. J. Bot. 88 (2001) 2013.

[2] B. Daizy, R.S. Harminder, K. Pal, R. Kumar, K. Shalinder, Forest Ecol. Manage. 256 (2008) 2166.

[3] O. Adeola, O. Nureni, O. Ekundayo, K. Wilfried, A, Flav. Fragr.J. 14 (1999) 241.

[4] K. Cimanga, K. Kambu, L. Tona, S. Apers, T. De Bruyne, N. Hermans, J. Totte, L. Pieters, A.J. Vlietinck, J. Ethnopharmacol. 79 (2002) 213.

[5] H. Ramezani, S. Harmider, B. Pal, R.K. Daizy, R. Kumar, Fitoterapia73 (2002) 261.

[6] P. Sartorelli, A.D. Marquioreto, A. Amaral-Baroli, M.E. Lima, P.R. Moreno, Phytother. Res. 21 (2007) 231.

[7] T. Takahashi, R. Kokubo, M. Sakaino, Lett. Appl. Microbial. 39 (2004) 60.

[8] M.H. Salari, G. Amine, M.H. Shirazi, R. Hafezi, M. Mohammadypour, Clin. Microbiol. Infect 12 (2006) 194.

[9] A. Kumar, Fitoterapia 59 (1998) 113.

[10] S. Benyahia, S. Benayache, F. Benayache, F. Leon, J. Quintana, M. Lopez, J.C. Estevez, F.J. Bermejo, Phytochemistry 66 (2005) 627.

[11] H.M. Ashour, Cancer. Biol. Ther. 7 (2008) 399.

[12] L.V. Sequist, B.A. Waltman, D. Dias-Santagata, S. Digumarthy, A.B. Turke, K. Bergethon, P. Fidias, A.T. Shaw, S. Gettinger, A.K. Cosper, S. Akavanfard, R.S. Heist, J. Temel, J.G. Christensen, J.C. Wain, T.J. Lynch, K. Vernovsky, E.J. Mark, M. Lanuti, A.J. Lafrate, M. Mino-Kenudson, J.A. Engelman, Sci. Transl. Med. 3 (2011) 75.

[13] W.D. Travis, E. Brambilla, A.P. Burke, A. Marx, A. G. Nicholson, World Health Organization Classification of Tumours. 4th ed. 7 (2015) 412.

[14] A.L. Tetlow, F. Tamanoi, Enzymes 33 (2013) 1.

[15] I.R. Vetter, A. Wittinghofer, Science 294 (2001) 1299.

[16] A. Hennig, R. Markwart, M.A. Esparza-Franco, G. Ladds, I. Rubio, Biol Chem. 396 (2015) 831.

[17] J.L. Bos, H. Rehmann, A. Wittinghofer, Cell. 129 (2007) 865.

[18] S. Jancik, J. Drabek, D. Radzioch, M. Hajduch, J. Biomed. Biotechnol. (2010) 1.

[19] C. Tan, X. Du. World J. Gastroenterol. 18 (2012) 5171.

[20] N.M.S. Walid, A. Mohamed, A.M. Dalal, S. Arun, A. Abdulaziz, A.M. Areej, A.M.E. Omar, PLOS ONE. 9 (2014) 11.

[21] A. Pender, I. Garcia-Murillas, S. Rana, R.J. Cutts, G. Kelly, K. Fenwick, I. Kozarewa, D. Gonzalez de Castro, J. Bhosle, M. O'Brien, N. C. Turner, S. Popat, J. Downward, PLOS ONE. 10 (2015) 1.

[22] E.L. Jackson, N. Willis, K. Mercer, R.T. Bronson, D. Crowley, R. Montoya, T. Jacks, D. A. Tuveson, Genes Dev. 15 (2001) 3243.

[23] P.M.K. Westcott, M.D. To, Chin. J. Cancer 32 (2013) 63.

[24] R.M. Mohammad, I. Muqbil, L. Lowe, C. Yedjou, H. Yin Hsu, L. Tzung Lin, M. David Siegelin, C. Fimognari, N.B. Kumar, Q. Ping Dou, H. Yang, A. K. Samadi, G. Luigi Russo, C. Spagnuolo, S.K. Ray, M. Chakrabarti, J.D. Morre, H.M. Coley, K. Honoki, H. Fujii, A.G. Georgakilas, A. Amedei, E. Niccolai, A. Amin, S.S. Ashraf, W.G. Helferich, X. Yang, C.S. Boosani, G. Guha, D. Bhakta, M. Rosa Ciriolo, K. Aquilano, S. Chen, S.I. Mohammed, W.N. Keith, A. Bilsland, D. Halicka, S. Nowsheen, A.S. Azmi, Semin Cancer Biol. 35 (2015) 78.

[25] Y.T. Peng, P. Chen, R.Y. Ouyang, L. Song, Apoptosis 20 (2015) 1135.

[26] A. Pérez-Garijo, Y. Fuchs, H. Steller, Elife 2 (2013) 1.

[27] A. Gross, J.M. McDonnell, S.J. Korsmeyer, Genes Dev .13 (1999) 1899.

[28] Z.-Y. Zhou, L.-L. Wan, Q.-J. Yang, Y.-L. Han, D. Li, J. Lu, C. Gheng, Toxicol. Lett. 259 (2016) 124.

[29] B. Alizadeh Behbahani, F. Tabatabaei Yazdi, A. Mortazavi, F. Zendeboodi, M.M. Gholian, A. Vasiee, J. Paramed. Sci. 4 (2013) 89.

[30] T. Mosmann, J. Immunol. Methods 65 (1983) 55.

[31] K.J. Livak, T.D. Schmittgen, Methods 25 (2001) 402.

[32] R. Dorantes-Heredia, J. Ruiz- Morales, F. Cano-Garcia, Transel Lung Cancer Res. 5 (2016) 410.

[33] AF. Gazdar, L. Girard, WW. Lockwood, WL. Lam, JD. Minna , J. Natl. Cancer Inst. 8 (2010) 1310.

[34] T. Lee, B. Lee, Y.-L. Choi, J. Han, M.-J. Ahn, S.-W. Um, J. Pathol. Transl. Med. 50 (2016) 197.

[35] SJ. Korsmeyer, Trends Genet. 11 (1995) 101.

[36] Y.-T. Hsu, R.J. Youle, J. Biol. Chem. 273 (1998) 10777.

[37] A. Gross, J. Jockel, M.C. Wei, S.J. Korsmeyer, EMBO J. 17 (1998) 3878.

[38] D.M. Finucane, E. Bossy-Wetzel, N.J. Waterhouse, T.G. Cotter, D.R. Green, J. Biol. Chem. 274 (1999) 2225.

[39] Z.N. Oltvai, C.L. Milliman, S.J. Korsmeyer, Cell 74 (1993) 609.

[40] T.W. Sedlak, Z.N. Oltvai, E. Yang, K. Wang, L.H. Boise, C.B. Thompson, S.J. Korsmeyer, Proc. Natl. Acad. Sci. U S A 92 (1995) 7834.

[41] EH. Cheng, B. Levine, L.H. Boise, C.B. Thompson, J.M. Hardwick, Nature 8 (1996) 554.

[42] C.M. Knudson, S.J. Korsmeyer, Nat. Genet. 16 (1997) 358.

[43] F. Islam, H. Khatun, S. Ghosh, M.M. Ali, J.A. Khanam, Asian Pac. J. Trop. Biomed. 2 (2012) 394.

[44] P.M. Doll-Boscardin, A. Sartoratto, B.H. Sales Maia, J.P. de Paula, T. Nakashima, P. Farago, C.C. Kanunfre, Hindawi Publishing Corporation (2012).

[45] M.J. Rathos, H.K. walkar, K. Joshi, S.M. Manohar, K.S. Joshi, BMC Cancer13 (2013) 29.

[46] R. Punia, K. Raina, R. Agarwal, R.P. Singh, PLOS ONE 12 (2017) 1.

[47] M.W. Anderson, S.H. Reynolds, M. You, R.M. Maronpot, Environ. Health Perspectives 98(1992) 13.

[48] J.L. Bos, E.R. Fearon, S.R. Hamilton, M. Verlaan-de Vries, J.H. van Boom, A.J. Van der Eb, B. Vogelstein, Nature 327 (1987) 293.

[49] J. Downward, Biochem. J. 376 (2003) 9.

[50] S. Schubbert, K. Shannon, G. Bollag, Nature Rev. Cancer 7 (2007) 295.

[51] T.A. Pham, I. Shair Mohammad, V.T. Vu, X.L. Hu, C. Birendra, A. Ulah, C. Guo, X.Y. Lü, W.C. Ye, H. Wang, Chem. Biodivers. 15 (2018) 1.

[52] S. Sharifi, J. Barar, M.S. Hejazi, N. Samadi, Adv. Pharm. Bull. 5 (2015) 351.

[53] T. Ranjbar, N. Asghari-Moghaddam, A. Mohammadgholi, Feyz, J. Kashan Univ. Med. Sci. 22 (2018) 442.