Research Article

THE ANTICANCER ACTIVITY OF JUGLONE VIA INDUCING PROGRAMMED CELL DEATH IN PANCREATIC CANCER CELLS

Dudu ERKOÇ KAYA[1], Duygu DURSUNOĞLU[2]

Abstract

Purpose: Pancreatic cancer (PC), has a poor prognosis with its silent course until the advanced level without any symptoms. Additionally, currently limited treatment options makes developing alternative treatment strategies and agents mandatory. In researches conducted to investigate new therapeutic agents for cancer, natural products are main sources. Among all, Juglone, a natural naphthoquinone, stands out with its cytotoxic effects in cancer cells, induction of apoptosis and autophagy, inhibition of antiangiogenesis and migration. Thus, in this study, we aimed to investigate the anticancer activity and the apoptotic effects of juglone in BxPC-3 pancreatic cancer cells.

Methods: BxPC-3 pancreatic cancer cells were treated with different doses of juglone. To evaluate the apoptotic effects following juglone treatments; proapoptotic *Bax*, antiapoptotic *Bcl-2* and an important inhibitor of apoptosis *Birc5 (Survivin)* gene expressions were determined by qPCR analysis. We also confirmed the results for apoptosis by performing immunofluorescence analysis using Annexin V-FITC.

Results: According to qPCR analysis, juglone induced statistically significant 2.24, 1.57, 2.43 fold increases of *Bax* gene expressions at 10, 15 and 20µM doses, respectively. We detected low fold increases after juglone treatments for *Bcl-2* gene expression at other treatments except 20µM treatment. These changes were not statistically significant. Juglone decreased *survivin* gene expression at all treatment doses while 2.39 fold decrease at 20µM doses of juglone was statistically significant. Also, we confirmed clearly by the immunofluorescence analysis that juglone increased apoptosis dose-dependently in BxPC-3 cells.

Conclusion: Taken together all the results of gene expression and immunfloresence analysis, our study suggests that juglone shows anticancer activity by inducing apoptosis possibly enhancing intrinsic apoptotic pathway in pancreatic cancer cells.

Keywords: apoptosis, *Bax* gene, *Bcl-2* gene, juglone, pancreatic cancer, *survivin* gene.

[1] Corresponding Author: Assist. Prof., Selçuk University, Faculty of Medicine, Department of Medical Biology, Konya, Türkiye dudu_erkoc@hotmail.com ORCID: 0000-0003-0114-6602

[2] Assist. Prof., Selçuk University, Faculty of Medicine, Department of Histology-Embryology, Konya, Türkiye ORCID: 0000-0003-4414-8659

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Juglonun Pankreas Kanseri Hücrelerinde Programlı Hücre Ölümünü İndükleyerek Gösterdiği Antikanser Etkisi

Öz

Amaç: Pankreas kanseri (PK), belirti vermeden ileri düzeye kadar sessiz seyri ile kötü bir prognoza sahiptir. Ek olarak, güncel tedavi seçeneklerinin oldukça sınırlı olması, alternatif tedavi stratejileri ve yeni teröpatik ajanların geliştirilmesini zorunlu kılmaktadır. Kanser için yeni terapötik ajanları araştırmak amacıyla yapılan araştırmalarda doğal ürünler temel kaynaklardır. Bunlar arasında doğal bir naftokinon olan Juglon, kanser hücrelerinde sitotoksik etkisi, apoptoz ve otofajiyi indüklemesi, antianjiyogenez ve kanser hücresi göçünü engellemesi ile öne çıkmaktadır. Bu nedenle çalışmamızda juglonun BxPC-3 pankreas kanseri hücreleri üzerindeki antikanser aktivitesini ve apoptotik etkilerini araştırmayı amaçladık.

Yöntem: BxPC-3 kanser hücrelerini farklı dozlarda juglon ile 24 saat muamele ettik. Juglon muamelesi sonrasındaki apoptotik etkileri değerlendirmek için; proapoptotik *Bax,* antiapoptotik *Bcl-2* ve önemli bir apoptoz inhibitörü olan *Birc5* (*Survivin*) genlerinin ekspresyon düzeylerini qPCR analizi ile belirledik. Annexin V-FITC ile immünofloresans analizi yaparak apoptoz sonuçlarını da doğruladık.

Bulgular: qPCR analizine göre juglon, 10, 15 ve 20µM dozlarda *Bax* gen ekspresyonlarında sırasıyla 2.24, 1.57 ve 2.43 kat düzeyinde istatistiksel olarak anlamlı artışlara neden oldu. *Bcl-2* gen ekspresyonu için, juglon tedavilerinden sonra 20µM doz dışındaki diğer uygulamalarda düşük kat artışları saptadık. Bu değişiklikler istatistiksel olarak anlamlı değildi. Juglon, tüm tedavi dozlarında *survivin* gen ekspresyonunu azaltırken, 20µM juglon uygulamasındaki 2.39 kat azalma istatistiksel olarak anlamlıydı. qPCR analizine ek olarak, juglonun BxPC-3 hücrelerinde doza bağlı olarak apoptozu arttırdığını immünofloresans analizi ile de doğruladık.

Sonuç: Gen ekspresyonu ve immünfloresans analizinin tüm sonuçları birlikte ele alındığında, çalışmamız juglonun pankreas kanseri hücrelerinde muhtemelen intrinsik apoptotik yolu indükleyerek antikanser aktivite gösterdiğini ortaya koymuştur.

Anahtar kelimeler: apoptoz, *Bax* geni, *Bcl-2* geni, juglon, pankreatik kanser, *survivin* geni.

1. INTRODUCTION

Despite increasing efforts to reduce risk factors in recent years, the prevalence of cancer is continuing to rise and is still one of the leading causes of death worldwide (You and Henneberg; 2017:140). Current standards include precise staging of cancer and chemotherapy, radiotherapy and/or surgical resection, although it is known to have significant side effects (Naidu et al., 2004:423). Pancreatic cancer (PC) is known as one of the most deadliest solid malignancies because of its clinically silent and aggressive characteristic (Bengtsson., 2020:16425). The current treatment of PC is very restricted as the chemotherapy with 5 Fluorouracil (5-FU) and nucleoside analogue gemcitabine, alone/in combination with radiation (Pereira and Corrêa., 2018:30). Additionally, the response of advanced pancreatic cancer patients to chemotherapy and radiotherapy treatment is quite low, making it an ongoing global health burden, with an overall 5-year survival rate of less than 5% among other cancer types (Bengtsson., 2020:16425, McGuigan., 2018:4846).

Thus, developing new treatmant agents and strategies for the battle with pancreatic cancer is inevitably necessary. Natural products and derivatives called herbal secondary metabolites (phytochemicals) constitute an important source of studies focused on investigating new therapeutic agents (Kitagawa et al., 2011:1084). Naphthoquinones are among the most active natural products obtained from plants and microorganisms. Naphthoquinones shows their biological activities through pleiotropic mechanisms such as reaction against cell nucleophiles, generation of reactive oxygen species and inhibition of some proteins. Juglone is a type of naphthoquinone known as 5-hydroxy-1,4-naphthoquinone and found in plants belonging to the Juglandaceae family. It is widely used for different aims, from using as a natural herbicide to a natural dye for fabrical products and food processing (Ravelo et al., 2003:719). Juglone has been reported to have special significance for cancer treatment due its significant role in the resistance of cancer cell proliferation, induction of apoptosis and autophagy in cancer cells, anti-angiogenesis process and repression of migration and invasion capacity of cancer cells, etc. as reviewed in Tang et al (2022:112785).

In this study we aimed to investigate the anticancer activity and the apoptotic effects of juglone in BxPC-3 pancreatic cancer cells. To evaluate the apoptotic effects; proapoptotic *Bax*, antiapoptotic *Bcl-2* and an important inhibitor of apoptosis *Birc5 (Survivin)* gene expressions were determined by qPCR analysis. We also confirmed the results for apoptosis by performing immunofluorescence analysis using Annexin V-FITC in pancreatic cancer cells.

2. MATERIAL AND METHODS

2.1. Cell Culture

BxPC-3 human pancreatic cancer cell line was supplied from the ATCC (Manassass, VA, USA). BxPC-3 cells were cultured in RPMI medium, containing 10% FBS with 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2. Tumor cells of three to ten passages in the actively growing condition and keeping their morphological and behavioral characteristics during culturing were used for the experiments.

2.2. Preparation of Juglone and Treatments of the Cells

Juglone were supplied commercially (Sigma-Aldrich Chemical Company, USA). Juglone was prepared in DMSO as 20 mM and stored at -20^{o}C. It was prepared freshly for every treatment. Juglone application doses were determined by considering the IC_{50} value as 21.05 for BxPC-3 cell line according to MTT analysis in our previous study (Avci et al., 2016:74). The determined doses were applied for 24 hours as 5 μM, 10 μM, 15 μM and 20 μM juglone.

2.3. RNA Isolation, cDNA Synthesis and Gene Expression Analyzes

Total RNA isolation from juglone treated and non-treated control BxPC-3 pancreatic cancer cells was carried out using TRIzol reagent. RNA quantification and quality was determined by spectrophotometer (NanoDrop, Thermo Fisher Scientific, USA). cDNA synthesis was performed using RT-PCR kit (RTPL12®, Vivantis, Malaysia). Evaluation of the expression levels of target genes *Bax*, *Bcl-2* and *survivin* were performed by quantitative real-time PCR (qPCR). The primers used in qPCR analysis are shown in Table 1. *β-actin* was used as reference gene for normalization. PCR reaction, performed using the Roche Light Cycler96, and were set as follows; an initial denaturation at 95°C for 5 min was followed by denaturation at 95°C for 30 sec recurrently at 40 cycles, annealing at 60°C for 30 sec, and elongation at 72 °C for 30 sec. All the analyzes were performed in triplicate. Relative changes in target genes were calculated as $2^{-\Delta\Delta CT}$ that is known as Livak method.

Table 1. Primers used in qPCR

Gene	**Primer sequence**
Bax	F: 5-GCTTCAGGGTTTCATCCAG-3 R: 5-CAGTTGAAGTTGCCGTCAGA -3
Bcl-2	F: 5-CCTGTGGATGACTGAGTACCTG -3 R: 5-TTCAGAGACAGCCAGGAGAAAT-3
Birc5 (*survivin*)	F: 5-AGGACCACCGCATCTCTACATTC-3 R: 5-CCTTGAAGCAGAAGAAACACTGGG-3
β-Actin	F: 5-ACT CTT CCA GCC TTC CTT C-3 R: 5-ATC TCC TTC TGC ATC CTG TC-3

2.4. Immunofluorescence Analysis for Apoptosis

Annexin V-FITC Apoptosis Detection Kit (Abcam, ab14085) was used to evaluate the apoptotic effect of the Juglone in BXPC-3 PC cells. In accordance with the manufacturer's recommendations; approximately 1-5x10^5 cells were seeded per well in a 24-well plate with a 12 mm coverslip in and left to incubate for 24 hours. After incubation, 5, 10, 15, and 20µM concentrations of Juglone were applied to the wells and allowed to incubate again for 24 hours. Following incubation, the Juglone medium was removed and 500µl of 1XBinding Buffer was added to each well. 5µl of Annexin V-FITC and 5µl of propidium iodide to separate necrotic cells from apoptotic cells were added to each well containing binding buffer. The plate placed on the mixer was incubated for 5 minutes at room temperature in a dark environment. Afterwards, the dye and buffer mixture were removed and the coverslips in the well were taken on the slide and prepared for evaluation. All the analyzes were performed in triplicate. Evaluation for apoptosis was done under fluorescent microscope (Olympus; BX51) and the images (Olympus; DP72) were recorded. Apoptotic cells in all groups were scored by dividing into 5 groups according to fluorescence staining intensity as; 0: negative staining, 1+: weak positive staining, 2+: moderately positive staining, 3+: strong positive staining and 4+: very strong positive staining.

2.5. Statistical Analysis

The data was indicated as means ± SD. Statistical analyses were performed by GraphPad Prism software (Version 8.0.2, San Diego, CA) and One-way ANOVA test was used for comparisons between groups. A $P < 0.05$ value was evaluated as statistically significant.

3. RESULTS

3.1. Cell Culture

In our study, human pancreatic cancer cell line BxPC-3 was used. During the culture studies, the cells were observed under an inverted microscope and determined that the cancer cells maintained their specific morphology and behavior.

3.2. Effects of Juglone on Gene Expressions

According to qPCR results after juglone treatment compared to the untreated group; juglone increased the expression of *Bax* gene strikingly 2.24, 1.57, 2.43 fold at 10, 15 and 20µM doses, respectively. All the fold increases were statistically significant (Figure 1).

The expression changes of *Bcl-2* gene in BxPC-3 cells compared to the control group after 24 hours of juglone application was detected as 1.9 and 1.46 and 1.02 fold increase at 5 µM, 10 µM and 15µM,

respectively while its expression was decreased 1.35 fold at 20 µM treatment (Figure 1). Changes were not statistically significant.

When we evaluated the expression changes of *survivin* gene after 24 hour juglone treatments, we determined that juglone caused decreases of 1.11, 1.4, 1.27, 2.39 folds in BxPC-3 cells. Of these, the 2.39 fold increase at 20 µM doses of juglone was statistically significant (Figure 1).

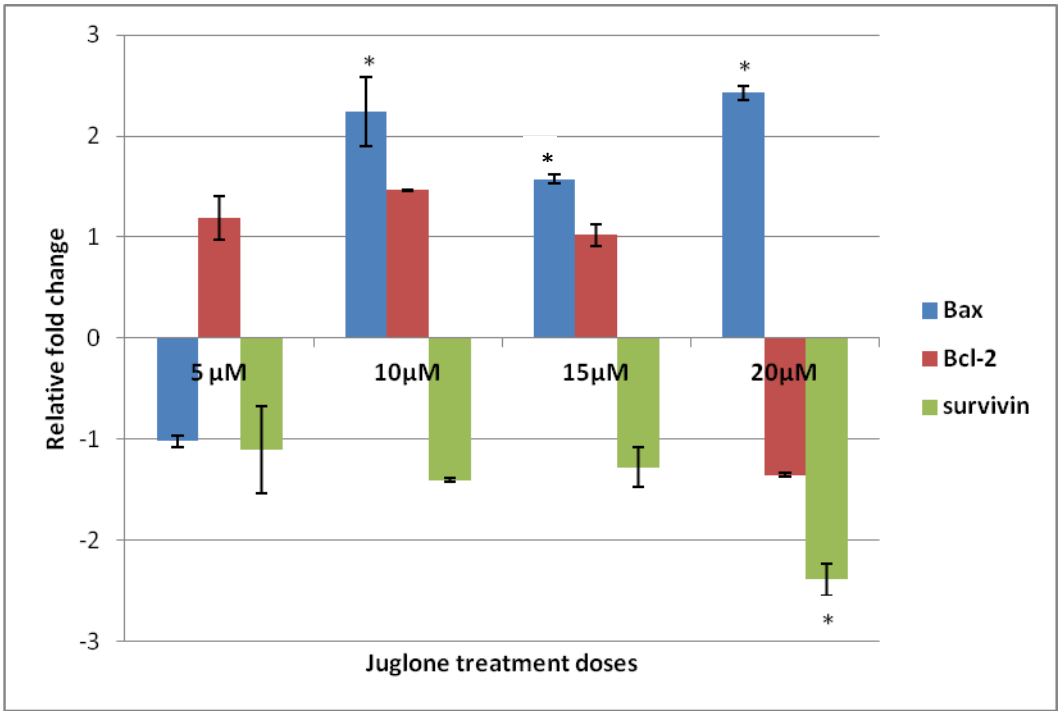

Figure 1. Effects of juglone on expression of *Bax*, *Bcl-2* and *Birc5* (survivin) genes in BxPC-3 PC cells. Values represent the mean ± SD from three independent experiments. *p< 0.05 compared to the control group.

3.3. Immunofluorescence Analysis for Apoptotic Effect of Juglone

According to the evaluation results of immunofluorescence apoptosis analysis using annexin V-FITC dye, juglone strikingly increased apoptosis dose-dependently in BxPC-3 pancreatic cancer cells (Figure 2).

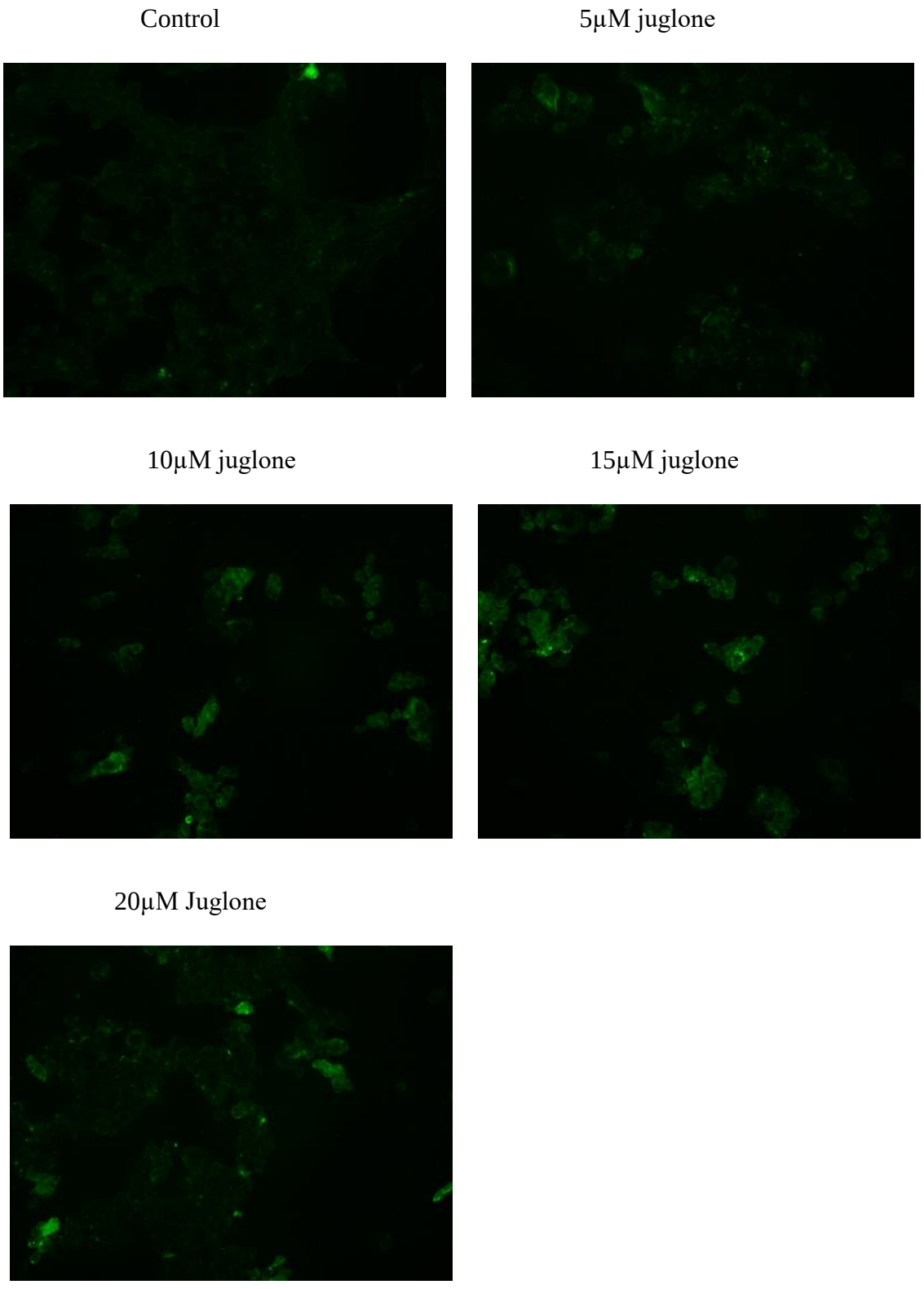

Figure 2. Immunofluorescence evaluation of the effects of juglone treatment on apoptosis in BxPC-3 pancreatic cancer cells. Annexin V-FITC staining. x40 magnification.

4. DISCUSSION

Cancer remains being a major burden all over the world. Although many anticancer agents have been identified, toxic effects and resistance impose as huge limits on their usage (Khan., 1999:96). As a reality, in the majority of cases, the side effects of current drugs hamper clinical outcomes. Thus, new agents characterised by better pharmaco-toxicological profiles are needed. Natural products are usually characterized by a better toxicological profile compared to traditional drugs. They can be used to increase the efficacy of traditional anticancer agents, and at the same time, to decrease their toxicity (Calcabrini et al., 2017:310, Catanzaro., 2018:469).

1,4-Naphtoquinones represent a class of molecules found in many plants, fungi, and bacteria which is characterised by an interesting anticancer profile (Verma., 2006:489). The main mechanism underpinning their antitumour potential is the generation of semiquinone radicals and other reactive oxygene specieses in the endocellular environment (Pavan et al., 2017:632). Juglone is a member of this family, and a naturally occurring compound found in the roots, leaves, fruit pericarp, bark and trunk of trees of the genus Juglans (walnut), used in Chinese, Indian and Korean traditional medicine (Xu et al., 2012:590) and acts as a growth-stunting agent (Kumar et al 2009) and apoptosis inducer (Catanzaro., 2018:469).

The apoptosis process works through different pathways described as the intrinsic or otherwise mitochondrial pathway and the other one is extrinsic or known as death receptor pathway. Intrinsic apoptotic pathway is regulated by Bcl-2 family which includes both pro- and anti-apoptotic members and the initiator of the intrinsic apoptosis is activation of caspase-9 (Ouyang et al., 2012:487). Conversely, the initiator of extrinsic apoptosis pathway is the activation of death receptors on the cell surface, leading to the activation of caspases-8, a significant marker in the extrinsic pathway. However, both pathways share a common downstream effector leading to cell death (Ouyang et al., 2012:487). Curiously, in previous studies, it has been reported that the anti-proliferative effect of juglone was mostly occured by inducing the mitochondrial-dependent apoptosis pathway. (Ji et al., 2011:69; Wang et al., 2019:40; Xu et al., 2012:590).

In our study, after treatment of BxPC-3 pancreatic cancer cells with juglone, we detected a significant increase of apoptosis when evaluated the expression levels of apoptosis-related genes *Bax*, *Bcl-2* and *survivin*. This significant effect of juglone on the increase of apoptosis in pancreatic cancer cells was also clearly confirmed by the immunofluorescence analysis. According to immunofluorescence analysis, juglone increased apoptosis dose-dependently in BxPC-3 PC cells.

Apoptosis process initiated by intrinsic pathway referring to mainly mitochondrial-mediated apoptotic pathway is regulated by the *Bcl-2*/*Bax* gene family. Some members of this family are inhibitors of apoptosis (antiapoptotic) and some induce apoptosis (proapoptotic) (Azmi et al., 2011:59). The intrinsic pathway is mediated by Bax/Bak insertion into mitochondiral membrane, subsequently, cytochrome c released from the mitochondrial intermembrane space into the cytosol (Kim 2005:1551). The cytochrome c combines with Apaf-1 and procaspase-9 to produce apoptosome which triggers caspase 9 following caspase signalling cascade that ends up to apoptosis (Jin and El-Deiry 2005:139; Los et al., 1995:81; Yuan and Akey., 2013:501). The *Bax* gene is induced by p53 and accelerates cell apoptosis. When the cell death signal arrives, *Bax* synthesis increases and it neutralizes the effect of *Bcl-2* and increases apoptosis (Oltvai et al., 1993:609). In our study, after juglone treatments prapoptotic *Bax* gene expression significantly increased at 10, 15 and 20 μM doses in BxPC-3 cancer cells.

Bcl-2 is the anti-apoptotic protein member of Bcl-2 family and prevents the release of cytochrome c. In several cancer types such as pancreatic, ovarian, lymphoma, multiple myeloma, lung, prostate adenocarcinoma, Bcl-2 overexpression was reported which can render cells resistant to chemotherapeutic agents. Thus, *Bcl-2* and related antiapoptotic proteins are common targets of new developing anti-cancer agents (Azmi et al., 2011:59). In our study, following juglone treatments, moderate increases of *Bcl-2* gene expressions was detected except 20μM dose that a moderate decrease of gene expression was detected. The results were not statistically significant.

Survivin, also known as *Birc5*, is an inhibitory protein of apoptosis and is expressed in a large number of malignancies (Altieri., 2003). Besides its anti-apoptotic properties, *survivin* protein also plays an important role in cell proliferation and angiogenesis (Andric et al., 2012:9), expressed in the G2/M phase of the cell cycle allowing cells to divide (Yazdani et al., 2012:100). Survivin expression levels are correlated with the aggresivity of the cancer and poor clinical outcome. Normal tissue cells express minimal *Survivin* expression while it is importantly much more in cancer tissues, thus, making it a critical target for tumour diagnostic and prognostic as well as anti-cancer therapies (Sah., 2006:164). In our study, juglone treatment caused a decrease in the apoptosis inhibitor *survivin* gene expression at all doses especially with a statistically significant level at 20 μM treatment in BxPC-3 cancer cells.

5. CONCLUSION

Pancreatic cancer is a challenging, agressive and lethal neoplasm showing poor prognosis and rapid metastases with limited curative options. Thus, researchers inevitably focuses to develop new anticancer agents and treatment strategies for effective fight against Pancreatic cancer. Natural

products and their seconder metabolites are the main sources of these investigations. Among them, as studied in our study, juglone is quite remarkable. Taken together all the results of gene expression analysis and the immunfloresence apoptosis evaluation in BxPC-3 pancreatic cancer cell line after juglone treatments, our study suggests that juglone shows anticancer activity by inducing apoptosis possibly enhancing intrinsic apoptotic pathway.

Supporting Organization

"There is no person/organization that financially supports the study."

Conflict of Interest

"The authors have no conflict of interest."

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