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journal homepage: www.elsevier.com/locate/btre*Peganum harmala* L.'s anti-growth effect on a breast cancer cell lineSomayeh Hashemi Sheikh Shabani^{a,1}, Sahar Seyed Hasan Tehrani^{a,1}, Zohreh Rabiei^a, Sattar Tahmasebi Enferadi^{a,*}, Gian Paolo Vannozzi^b^a National Institute of Genetic Engineering and Biotechnology, Tehran, Iran^b Dipartimenti di Scienze Agraria, Università degli Studi di Udine, Udine, Italy

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ABSTRACT

This research was done to evaluate the induction of apoptosis in MDA-MB-231 breast cancer cell line by *Peganum harmala*'s extract, in which a significant amount of β -carbolines is included. The apoptosis incidence was assessed through Annexin-V-Fluorescence kit. The expressions of genes through which intrinsic apoptosis pathway are involved, Bax, Bcl-2, Bid, and Puma, over the genes the expressions of which are linked to extrinsic apoptosis pathway, TRAIL, Caspase8, p21, and p53, were examined by RT-PCR and Real-time PCR. The results demonstrate that the extract decreases the growth rate of the cancer cell line through inducing apoptosis mechanism. As long as the expression of anti-apoptosis Bcl-2 gene reduced dramatically, an over-expression in Bax and Puma genes was monitored indicating activation of intrinsic apoptosis pathway. A notable over-expression observed with TRAIL and Caspase8 genes as well as Bid gene. The latter is an intermediate for both intrinsic and extrinsic pathways of apoptosis.

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1. Introduction

Apoptosis is a programmed cell death which is important in controlling cell number and proliferation. Apoptosis induces by two major pathways; extrinsic and intrinsic pathways [8]. Extrinsic pathway instigates by death signals that ligate to the death receptor such as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) or Fas (TNF Receptor Superfamily) genes. The death receptors are the member of the tumor necrosis factor (TNF) receptor gene superfamily containing death domain which is important in transmitting the death signal from the cell's surface to intracellular signaling pathways. The ligation of death receptor and death signal caused receptor trimerization, recruiting adaptor molecules such as FADD (Fas-Associated protein with Death Domain) which results in the activation of the initiator Caspase-8 and lead to activate downstream effector caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases) such as caspase-3 and finally DNA fragmentation [9,3].

The other pathway, intrinsic or mitochondrial pathway is triggered by the release of apoptosis factors such as cytochrome c. The Bcl-2 (B-cell lymphoma 2) family proteins are crucial for

intrinsic pathway that inhibit or initiate apoptosis. Antiapoptotic Bcl-2 family members such as Bcl-2 and Mcl-1 inhibit apoptosis; however the domain of proapoptotic members such as Bax (Bcl-2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer) promote apoptosis and also suppress oncogenesis. Bid (BH3 interacting-domain death agonist), a Bcl-2 family protein with a BH3 domain only, is a linkage between intrinsic pathway and extrinsic pathway [9,8].

Evading apoptosis is one of the hallmark of cancer cells [13,14]. The researchers postulated that inducing apoptosis in cancer cells which is causing no damage to normal cells can be a practical drug for treating cancer. Therefore, ongoing cancer therapies are looking for the anti-cancers triggering apoptosis in cancer cells [10,3,9].

Peganum harmala L. (*Nitrariaceae*) is a local remedy distributed in the central Asia, Middle East and North Africa. This plant has been used as folk medicine mostly because of its antibacterial effect for long time. Studies show the inhibitory effects of *P. harmala* on bacteria, parasites, viruses, and cancer cells [2]. Several reports demonstrated that β -carboline alkaloids, one of the phytochemical compound of *P. harmala*, intercalate to DNA leads to DNA topoisomerases inhibition [1]. That study addressed that harmaline and harmalol induce melanogenesis through p38 MAPK signaling [17]. The researchers suggested the anti-cancer effect of *P. harmala* happens mainly as a consequence of inducing apoptosis [4,11]. Indeed, the impact of harmine on B16f-10 melanoma revealed that it activates both intrinsic and extrinsic pathways by up-regulating Bax, Bid, p53 and Caspase-8 genes and down-regulating Bcl-2 [11]. By contrast, harmine and its derivatives have

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no effect on the expression of *p53* and *Bax* when cells treated with *P. harmala*. The over-expression of *Fas* and down-regulation of *Bcl-2* in cancer cells treated with harmine/its derivatives was observed, too [6]. Cao et al. [4], have reported that expression of *Bcl-2*, *Mcl-1* (induced myeloid leukemia cell differentiation protein *Mcl-1*) and *Bcl-xl* (B-cell lymphoma-extra large) genes were reduced in HepG cancer cells while the expression of *Bax* didn't change suggesting that it induces intrinsic pathway in apoptosis [4]. Chen et al. [7] used 9 harmine derivatives (including harmine) to investigate their antitumor effects and acute toxicities in mice through the analyses of IC50 and the expression of *Bcl-2* gene. However, they stated that further studies on the effects of harmine derivatives on key regulators for tumor cell apoptosis were needed. Hamsa and Kuttan [12] have demonstrated the anti-angiogenic activity of harmine using *in vivo* and *in vitro* assay systems and concluded strong angiogenic inhibitory of harmine with the ability to decrease the proliferation of vascular endothelial cells and reduction in the expression of various pro-angiogenic factors. Zhao and Wink [21] have studied harmine activity on telomerase by analyzing the telomeric repeat amplification protocol (TRAP) and demonstrated significant inhibition of telomerase activity and an induction of an accelerated senescence phenotype by over-expressing elements of the *p53/p21* pathway as a result of harmine treatment which exhibits a pronounced cytotoxicity on cancer cells. Sun et al. [19] and Zhang et al. [20] have studied the effects of paclitaxel and/or harmine on the cell migration and invasion in two human gastric cancer cell lines and it was elucidated that those compounds provide a synergistic effect on growth inhibition of cancer cells via the downregulation of Cyclooxygenase-2 (COX-2) expression.

Despite all the aforementioned evidences, the underlying mechanism of harmine effect on cancer cell line most of which is not clear yet has not been thoroughly supported.

In the previous study, the anti-cancer impact of *P. harmala* on cancer cell lines was confirmed by MTT test [18]. In the present study, the apoptosis assay was used to elucidate the anticancer effect of *P. harmala*'s seed extract. The expression of *Bax*, *Bcl-2*, *Bid* and *Puma* (*p53* upregulated modulator of apoptosis) genes being involved in intrinsic pathway has been studied as some related mechanisms of *P. harmala*'s extract. Furthermore, the gene expression of *TRAIL* and *Caspase-8* as candidates genes involved in extrinsic apoptosis pathway was monitored. For further confirmation, *p53* and *p21* were traced using quantitative procedures, real-time PCR.

2. Materials and methods

2.1. Cell culture and preparation

The breast cancer cell line MDA-MB-231, was obtained from National Institute of Genetic Engineering and Biotechnology, NIGEB. The cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin ($100 \mu\text{mL}^{-1}$), and streptomycin ($100 \mu\text{mL}^{-1}$) at 37°C in a humidified atmosphere containing 5% CO_2 maintained.

2.2. Chemicals

P. harmala seeds were collected from the mountains of Shahrekord, Chaharmahal Bakhtiari province, Iran. The *P. harmala*'s alkaloid extract has been prepared according to Seyed Hasan Tehrani et al. [18].

Annexin-v-fluos staining Kit (Roche Applied Science, France), RNA extract kit (Roche Applied Science, France), RNeasy Mini Kit Qiagen (Cat. No. 74104), Master Mix PCR (1 intron), QuantiFast SYBR Green (Qiagen, Cat. No. 204054), Harmine (286044-1G, Sigma, USA), Harmaline (51330-1G, Sigma, USA) and DMEM, Trypsin, and FBS (bio idea, Italy) were used for our experiments.

2.3. Apoptosis assay

Annexin-v-fluos staining Kit used for differentiating apoptotic cells from necrotic cells in the early stage of apoptosis by binding to the cells including phosphatidyl serine. Cancer cell line MDA-MB-231 were seeded (1000 cells/well) in 24 well plate and incubated for 24 h at 37°C in 5% CO_2 atmosphere. The concentration of 30 and $100 \mu\text{g.mL}^{-1}$ of *P. harmala*'s seed extract was added and incubated for further 24 and 48 h. After incubation the medium depleted, for staining with annexin-V, each well was incubated for 15 min at 25°C in $100 \mu\text{l}$ of a solution containing $20 \mu\text{l}$ of Annexin-V and $20 \mu\text{l}$ propidium iodide ($30 \mu\text{g.mL}^{-1}$) in 1 ml HEPES buffer (10 mM HEPES/NaOH pH 7.4; 140 mM NaCl; 5 mM CaCl_2). Cells were washed twice with phosphate-buffered saline and analyzed under a fluorescence microscopy using an excitation wavelength of 480 nm and detection in the range of 515–565 nm.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from MDA-MB-231 cells treated with $30 \mu\text{g.mL}^{-1}$ concentration of alkaloid extract for 24 h (as the

Table 1

List of primer pairs used correlated with apoptosis pathway in human cells.

Accession no. ^a	Target Gene	Sequence (5' → 3')	Length sequence ^b	
NM_001199954.1	β -actin	F R	AAGGACTCTATAGTGGGTGACGA ATCTTCTCCATGTCGTCCAGTTG	107
NM_138764.4	<i>Bax</i>	F R	TGCCTCAGGATGCGTCCACCAA CCCCAGTTGAAGTTGCCGTCAG	175
NM_000633.2	<i>BCL-2</i>	F R	CGGAGGCTGGGATGCTTTGT CAAGTCCCACCAGGGCCAAA	111
NM_001244572.1	<i>Bid</i>	F R	CTTGCTCCGTGATGCTTTTC TCCGTTCACTCCATCCCATTT	100
NM_014417.4	<i>Puma</i>	F R	GACGACCTCAACGCACAGTA AGGAGTCCCATGATGAGATTG	101
NM_001080125	<i>Caspase-8</i>	F R	ATTAGGGACAGGAATGGAACAC GGAGAGGATACAGCAGATGAAG	180
NM_001190942.1	<i>TRAIL</i>	F R	CCGTCAGCTCGTTAGAAAGACTCCA GCAAGTTGCTCAGGAATGAATGCC	103
NM_001126118.1	<i>p53</i>	F R	GAGTTGGCTCTGACTGTACC TCCGTTCCAGTAGATTACCAC	133
NM_001220778	<i>p21</i>	F R	GGAGACTCTCAGGGTCCGAAAAC GGGCTTCTCTTGAGAAGATC	90

minimum lethal dose that kills approximately 50% of cells occurred at 24 h (IC50) obtained by MTT test, this combination of time/concentration has been chosen for the rest of analysis) using the RNA extraction Kit (RNeasy Mini Kit Qiagen (Cat. No. 74104)) according to the manufacturer's protocol. The quality of the RNA samples and concentration were precisely determined using NanoDrop spectrophotometer. RNA reverse transcription was performed in a final volume of 20 μ l using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Cat. No. 6110, Iran).

2.5. Primer design

Upon a survey on NCBI GenBank, the related sequences for *Bax*, *Bcl-2*, *Bid* and *Puma*, *TRAIL* and *Caspase-8* involved in apoptosis pathway and also *p53* gene were selected and the appropriate primer pairs were designed with lengths ranging from 70 to 186 bp

with the programs Oligo 6.0. BLASTN searches conducted against nr (the nonredundant set of GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen for the primers. To avoid amplification of contaminating genomic DNA, one of the two primers or the probe was placed at the junction between two exons or in a different exon. Designed primers were purchased from Gene fanavaran (Iran). The primers details are reported in Table 1.

2.6. RT-PCR

Reverse transcriptase reactions contained 20 ng of RNA samples, 50 nM RT random hexanucleotide primers (Amersham Pharmacia Biotech, Piscataway, NJ), 1 \times RT buffer (10 mM Tris-HCl, 0.1 M KCl, 0.05% [wt/vol] Tween 20, 0.75 mM EGTA [pH 8.3], 2 mM deoxyribonucleoside triphosphates, 2.5 mM MgCl₂, 3.3 U. μ l⁻¹

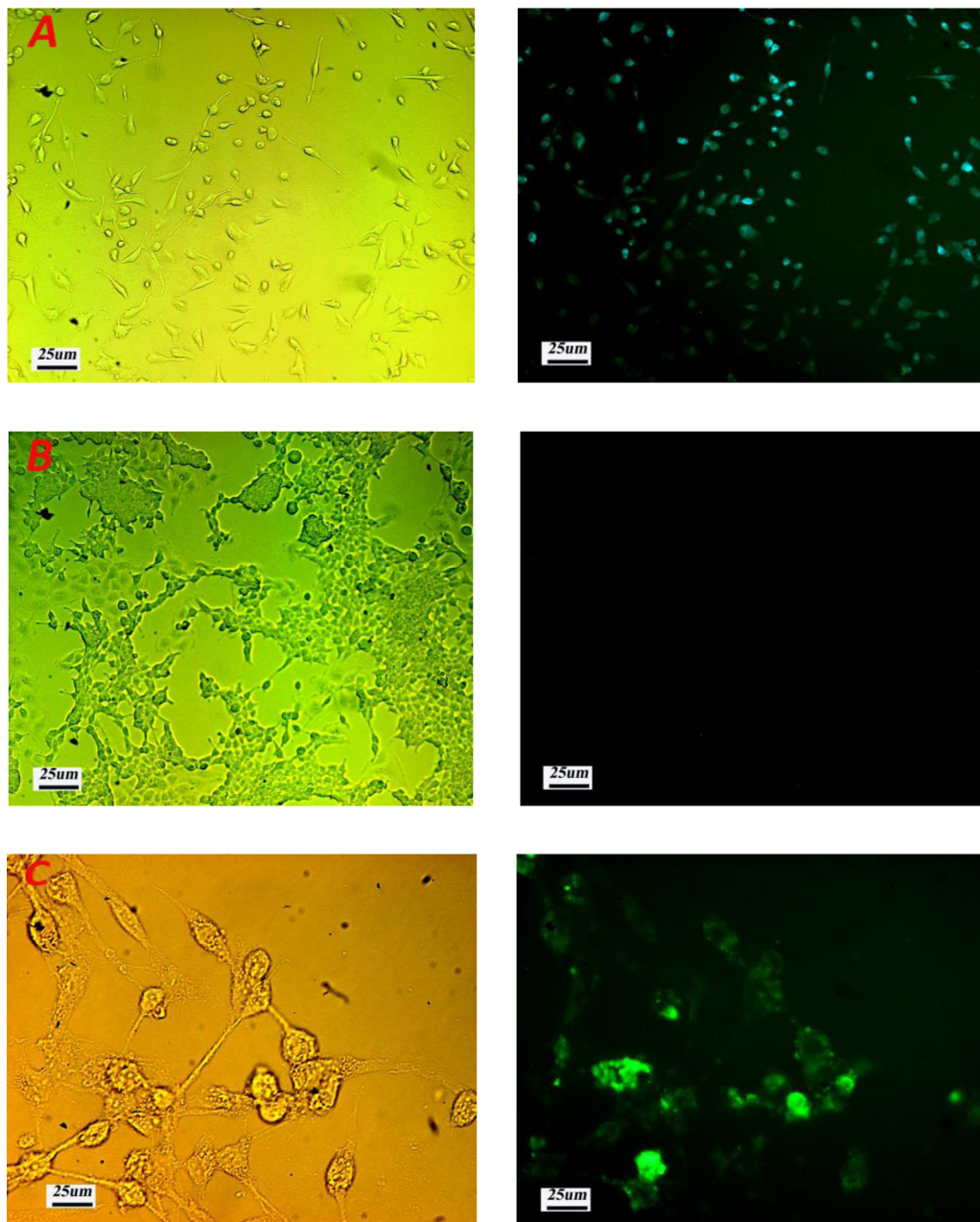


Fig. 1. Annexin-v-fluos staining of MDA-MB-231 cells treated with 30 μ g ml⁻¹ of *P. harmala* L.'s seed extract. (A) after 24 h of exposure (cells emitted green under excitation light), (B) untreated cells as control, and (C) cells after 48 h of exposure. The right mirrors of each micrograph is negative of its related one.

reverse transcriptase and $0.5 \text{ U} \cdot \mu\text{l}^{-1}$ RNasein (all purchased from cDNA Archive kit of Applied Biosystems). The reaction mixture was sequentially incubated in an Applied Biosystems 9800 Thermo-Cycler for 30 min at 16°C , 30 min at 42°C , 5 min at 85°C . The reverse transcription reaction was stopped by heating to 95°C for 5 min. Next, 0.5 U of RNase H (Invitrogen) was added, and the reaction mixture was incubated at 37°C for an additional 30 min. Amplified PCR (15 ml) products were electrophoresed on a standard 1.8% agarose gel having stained with ethidium bromide and photograph under UV light.

2.7. Real-time quantitative PCR (qPCR)

The synthesized cDNA was quantified by the QuantiFast SYBR Green (Qiagen, Cat. No. 204054) according to the manufacturer's protocol. For each PCR run, $1 \mu\text{l}$ primer ($10 \mu\text{M}$) and $300 \text{ ng} \cdot \mu\text{l}^{-1}$ cDNA were added to the PCR master mix and reached to the final volume of $20 \mu\text{l}$. Real-time PCR was performed with an ABI Prism 5700 or ABI Prism 7700 machine (Applied Biosystems, USA) and universal cycling conditions (2 min at 50°C , 10 min at 95°C , 40 cycles of 15 s at 95°C , and 1 min at 60°C). Experiments were performed with duplicates for each data point. No template was added to negative control reactions. In order to quantify specific gene expression, the mRNA level in each sample was calculated relative to beta-actin. The Ct data was determinate using default threshold settings. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The relative quantification of gene expression was analyzed by the $\Delta\Delta\text{Ct}$ method. The expected size of PCR products and not having primer dimer were confirmed by gel electrophoresis on a standard 1.2% agarose gel stained with ethidium bromide and visualized under UV light.

2.8. Statistical analysis

The reaction efficiencies were estimated using the LinReg tool for each sample, the expression ratios were tested by random pairwise reallocation using REST software.

3. Results

3.1. Apoptosis analysis

Apoptosis, a normal phenomenon, through which a suicide program is activated within the cell occurs frequently in multicellular organisms, however is associated with DNA fragmentation, cytoplasm shrinkage, membrane changes and finally cell death without releasing harmful substances into the surrounding area.

Upon treating MDA-MB-231 cancer cell line with *P. harmala* L.'s seed extract several changes were observed depending the concentration and time of exposure of which $30 \mu\text{g} \cdot \text{ml}^{-1}$ concentration of the extract lead to a significant growth rate reduction and the minimum lethal dose that kills approximately 50% of cells at 24 h (IC50). Providing more deleterious sign for the later concentration was responsible for smaller cell size and more shrunk cytoplasm (data are not shown).

3.2. Annexin-v-fluos assay

To validate apoptosis pathway induction and cell death occurrence as its consequence, Annexin-v-fluos assay was used. Cancer cell line MDA-MB-231 were treated with $30 \mu\text{g} \cdot \text{ml}^{-1}$ concentration of *P. harmala* L.'s seed extract and were observed under fluorescence light after 24 and 48 h. The 100% percentage of

treated cells was fluorescent green while untreated cells didn't emit any green light (Fig. 1).

3.3. Gene expression analysis

The quantitative and qualitative analyses of gene expression by which a reliable calculation of the gene functions in a cell get evidenced need to be traced. Thus, the expressions of the genes involved in intrinsic apoptosis pathway namely, *Bax*, *Bcl-2*, *Bid*, and *Puma* as well as the genes involved in extrinsic apoptosis pathway such as *TRAIL* and *Caspase-8* and two more genes, *p21* and *p53*, were evaluated qualitatively and quantitatively by RT-PCR and Real-time PCR, respectively.

In order to investigate mRNA differential expression in MDA-MB-231 cancer cell line, the impact of *P. harmala* L.'s seed extract ($30 \mu\text{g} \cdot \text{ml}^{-1}$ /24 h) were conducted as well.

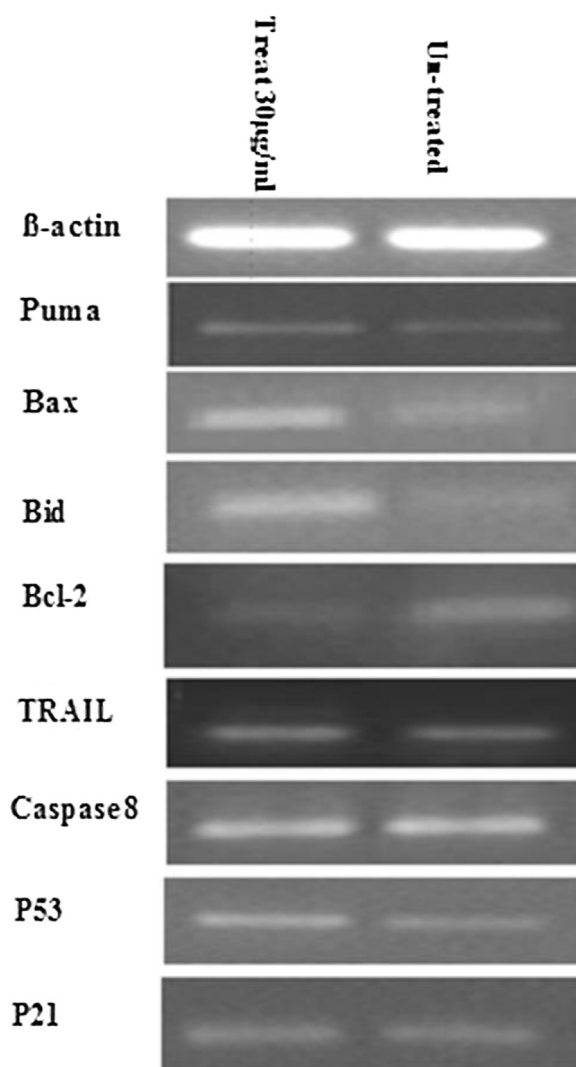


Fig. 2. Impact of *P. harmala* L.'s seed extract on the expression of different genes. MDA-MB-231 cells were treated with $30 \mu\text{g} \cdot \text{ml}^{-1}$ of the extract for 24 h. Expression of beta-actin was used to normalize the conditions. *PUMA*: p53 upregulated modulator of apoptosis, *Bax*: *Bcl-2*-associated X protein, *Bid*: *BH3* interacting-domain death agonist, *Bcl-2*: B-cell lymphoma 2, *TRAIL*: tumor necrosis factor-related apoptosis-inducing ligand, *caspases*: cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases, *p53*: phosphoprotein p53 or tumor suppressor p53, *p21*: cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1.

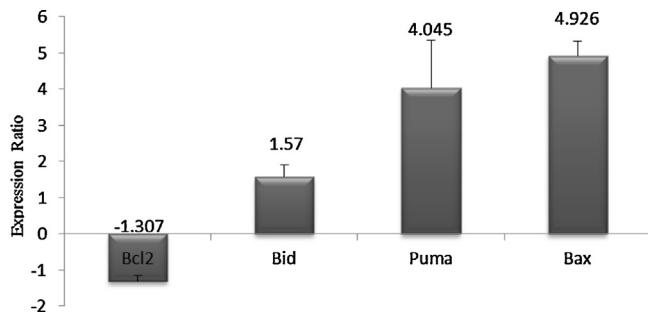


Fig. 3. Intrinsic apoptosis-related gene expression in cells treated by *P. harmala* L.'s seed extract. Pro-apoptotic genes, *Bax*, *Puma*, and *Bid*, were up-regulated while the anti-apoptotic gene, *Bcl-2* showed less expression compare to control. *Bcl-2*: B-cell lymphoma 2, *Bid*: *BH3* interacting-domain death agonist, *PUMA*: p53 upregulated modulator of apoptosis, *Bax*: (*Bcl-2*-associated X protein).

Fig. 2 shows changes in the expression of eight genes involving apoptosis pathway compared to a housekeeping gene (beta-actin) in MDA-MB-231 cell line under two conditions, being treated with *P. harmala* L.'s seed extract and keeping the cells untreated. RT-PCR result showed that the expression of pro-apoptotic genes *Puma*, *Bax*, and *Bid* involving in intrinsic pathway increased significantly in comparison with control, while the anti-apoptotic gene *Bcl-2* diminished. Meanwhile the extrinsic gene *TRAIL* and *Caspase8* were up-regulated, also the expression of the intermediate gene in both intrinsic and extrinsic pathways, *Bid*, increased meaningfully. The figure also reveals the overexpression in *p53* and *p21* genes compared to their control (Fig. 2).

Accordingly, in real-time PCR analysis, the expression of pro-apoptotic genes, *Bax*, *Puma*, and *Bid*, increased 4.926, 4.045, 1.57 times, respectively, despite the expression of *Bcl-2* decreased (1.307 times) when compared with its respected control (Fig. 3). Further studies on the extrinsic gene expression demonstrated that the death receptor genes, *TRAIL* and *Caspase-8* were up-regulated rising to 1.571 and 1.867 times, respectively (Figs. 4 and 5). However, the highest over-expression is dedicated to *p53* gene (3.938). Additionally, the expression of *p21* gene rose to 1.64 time in the treated cells (Fig. 4).

4. Discussion

The anti-cancer effect of *P. harmala*'s seed extract on decreasing the growth rate of cancer cells has been approved according to our previous study. In the present study, we scrutinized this impact at molecular level through following up the expression of some related genes. The morphological changes including damaged cell membrane and cell shrinkage are the features of apoptosis as

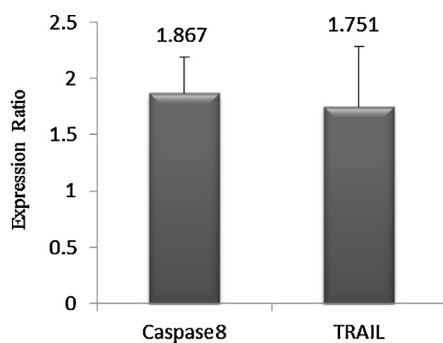


Fig. 4. Extrinsic apoptosis-related gene expression in cells treated by *P. harmala* L.'s seed extract. *TRAIL* and *Caspase-8* genes expression increased.

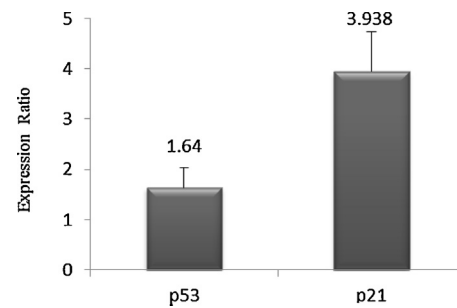


Fig. 5. Apoptosis and growth related gene expression in cells treated by *P. harmala* L.'s seed extract. The *p53*, *p21* genes expression were significantly elevated. *p53*: phosphoprotein p53 or tumor suppressor p53, *p21*: cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1.

suggested by [16] on cancer cell death caused by apoptosis pathway. This was confirmed by Annexin-v-fluorescence assay as well. The treated cells all of which were green under fluorescence light (and obviously not red) were damaged into their nucleolus. This means that apoptosis triggers death in cancer cells and leaves out the necrosis as an option. This phenomena would be intensified by increasing the exposure time (from 24 h to 48 h) and the most deleterious cell death was observed at 48 h as shown in Fig. 1 (the mirrors of 1A and 1C micrographs).

It is noteworthy that apoptosis is mainly induced by two pathways; extrinsic and intrinsic. Admittedly, in this study we examined the effect of *P. harmala* L.'s seed extract through which apoptosis pathway are induced. As a result of the fact that extrinsic pathway gets triggered by ligation of death signal and receptors, *Caspase-8* gene is activated as a consequence of intracellular signaling. The active *Caspase-8* initiate *Caspase* cascade the role of which on morphology of apoptosis is not clear [8].

The expression of two genes *TRAIL*, death receptor, and *Caspase-8*, associated with extrinsic pathway, which were followed by RT-PCR and Real-time PCR demonstrated up-regulation of both genes upon treating with *P. harmala* L.'s seed extract suggesting that the extract induces mainly on apoptosis extrinsic pathway.

On the other hand, to identify the cooperation of intrinsic pathways in anti-cancer effect of *P. harmala* L.'s seed extract, *Bcl-2* gene family was mainly followed up. The pro-apoptotic genes, *BH3* multidomain, *Bax* (*Bcl-2*-associated X protein) and *Bak* (*Bcl-2* homologous antagonist/killer) caused permeability in mitochondrial membrane which was inhibited by anti-apoptotic proteins, *Bcl-2*, *Bcl-xl*. As a consequence of binding *BH3*-only proteins (*BID*, *BIM* (*Bcl-2*-like protein 11), *BAD* (*Bcl-2*-associated death promoter), and *PUMA* anti-apoptotic proteins, their activities were inhibited. However, in this research *Bcl-2* as an anti-apoptotic protein, *Bax* as a *BH3* multidomain pro-apoptotic protein and *PUMA* as *BH3*-only domain pro-apoptotic protein were selected for evaluation of related genes affecting the intrinsic pathway. *Bid* also studied not only as a pro-apoptotic protein but also as linkage between intrinsic pathway and extrinsic pathway [8].

Expression analysis of mRNA by RT-PCR and Real-time PCR revealed that expression of antiapoptotic gene, *Bcl-2* decreased in treated cell in compare to control while the pro-apoptotic genes such as *Bax*, *Puma*, *Bid* increased. All the results support each other and propose that the *P. harmala*'s seed extract induces intrinsic pathway when exposed to cell lines.

The expression changes of *p53* and *p21* in MDA-MB-231 cancer cell line treated with *P. harmala*'s seed extract were also studied. Both *p53* and *p21* were up-regulated in treated cells in compared with their respected control. Accordingly, it approved that *P. harmala*'s seed extract decreases the cell growth rate, hence *p53* as transcription regulator up-regulates *Bax*, *Puma* and *TRAIL* [15,5].

5. Conclusion

Cancer cells grow and divide further from normalcy due to losing the general controls over the growth. Traditional treatments involve diminishing the growth rate of cancer cells by means of exposing it against a medicinal herb extract. In the present study, *P. harmala* L.'s seed extract exposed on a MDA-MB-231 cancer cell line and its growth inhibition was followed through both morphological changes observation and following genes involved programme cell death.

P. harmala L.'s seed extract induced cell death and decreased the cell growth in the breast cancer cell line. The cell death was caused by apoptosis which was triggered by both intrinsic and extrinsic pathways which suggest that herb might be useful for preventing the development of tumors.

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