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Peganum harmala L.'s anti-growth effect on a breast cancer cell line

Somayeh 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 Sienze Agraria, Universita 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 ß-carbolines is included. The apoptosis incidence was assessed through Annexin-V-Flous 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 gen 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.

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 (*c*ysteine-*asp*artic prote*ases* or **c**ysteine-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 ß-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

* Corresponding author at: National Institute of Genetic Engineering and Biotechnology, Tehran freeway 15 km, P.O.BOX 14925/16, Pajouhesh BLV 1417863171, Tehran, Iran. Fax: +98 2144787399.
E-mail address: tahmasebi@nigeb.ac.ir (S. Tahmasebi Enferadi).
[1] The first two authors are considered as the first author.

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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 ($100\,\mu mL^{-1}$), and streptomycin ($100\,\mu mL^{-1}$) 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, Chaharmahalo 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 (I 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 $100\,\mu g.ml^{-1}$ 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 μl of a solution containing 20 μl of Annexin-V and 20 μl propidium iodide ($30\,\mu g\,ml^{-1}$) 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 $30\,\mu g.ml^{-1}$ 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	AAGGACTCCTATAGTGGGTGACGA	107
		R	ATCTTCTCCATGTCGTCCCAGTTG	
NM_138764.4	*Bax*	F	TGCCTCAGGATGCGTCCACCAA	175
		R	CCCCAGTTGAAGTTGCCGTCAG	
NM_000633.2	*BCL-2*	F	CGGAGGCTGGGATGCCTTTGT	111
		R	CAAGCTCCCACCAGGGCCAAA	
NM_001244572.1	*Bid*	F	CTTGCTCCGTGATGTCTTTC	100
		R	TCCGTTCAGTCCATCCCATTT	
NM_014417.4	*Puma*	F	GACGACCTCAACGCACAGTA	101
		R	AGGAGTCCCATGATGAGATTG	
NM_001080125	*Caspase-8*	F	ATTAGGGACAGGAATGGAACAC	180
		R	GGAGAGGATACAGCAGATGAAG	
NM_001190942.1	*TRAIL*	F	CCGTCAGCTCGTTAGAAAGACTCCA	103
		R	GCAAGTTGCTCAGGAATGAATGCCC	
NM_001126118.1	*P53*	F	GAGGTTGGCTCTGACTGTACC	133
		R	TCCGTCCCAGTAGATTACCAC	
NM_001220778	*p21*	F	GGAGACTCTCAGGGTCGAAAAC	90
		R	GGGCTTCCTCTTGGAGAAGATC	