Growth Inhibitory Impact of *Peganum harmala* L. on Two Breast Cancer Cell Lines

Sahar Seyed Hassan Tehrani¹,§; Somayeh Hashemi Sheikh Shabani¹,§; Sattar Tahmasebi Enferadi¹; Zohreh Rabiei¹

¹National Institute of Genetic Engineering and Biotechnology, Tehran, IR Iran
§Corresponding author: Sattar Tahmasebi Enferadi, National Institute of Genetic Engineering and Biotechnology, Tehran freeway 15 Km, Pajouhesh BLV, Zip Code: 1417863171, P. O. Box 14925/16, Tehran, IR Iran. Tel: +98-2144580429, Fax: +98-2144580399, E-mail: tahmasebi@nigeb.ac.ir

Received: May 24, 2013; Revised: October 10, 2013; Accepted: March 3, 2014

**Background:** 8-carbolines, harmaline and harmine, are major alkaloids present in the seeds of *Peganum harmala* L. These alkaloids are known as herbal active principals with potential use in pharmaceutical and medicine.

**Objectives:** To assess the growth inhibitory effect of phyto-alkaloids, harmaline and harmine, on cancer cell lines.

**Materials and Methods:** *P. harmala* L’s alkaloids were extracted by acidic/basic extraction method and identified by two methods, Fourier Transform Infra-Red Spectroscopy (FTIR) and High Performance Liquid Chromatography (HPLC). Two breast cancer cell lines, MDA-MB-231 and MCF-7, were subjected to different concentrations (1–100 μg.mL⁻¹) of the *P. harmala* extract at different time courses (24, 48, and 72 hours). Methylthiazol Tetrazolium (MTT) test, half maximal inhibitory concentration (IC₅₀) and morphological changes through optical microscopy were evaluated.

**Results:** In both studied cell lines, the *P. harmala* extract decreased cell viability in longer time exposure in a dose dependent manner. The more concentrated extract led to higher motility of MDA-MB-231 at 24 hours. Although, MCF-7 cell line required longer exposure time to reach the same motility. It was observed that 30 μg.mL⁻¹ is the minimum lethal dose that kills approximately 50% of cells at 24 hours for MDA-MB-231 cell line (IC₅₀). IC₅₀ for MCF-7 was calculated 40 μg.mL⁻¹ and 25 μg.mL⁻¹ 48 and 72 hours after being exposed against harmala’s extract, respectively. The morphological observation confirmed the apoptosis nature of *P. harmala* on cells as their membrane kept intact and no membrane permeabilization was observed.

**Conclusions:** The results revealed that the *P. harmala* extracts significantly decreased the growth rate and cell survival of cancer cell lines. The extract induced cell death regarding natural cell growth rate. MDA-MB-231 cell line naturally has a higher growth rate than MCF-7 cell line, thus higher growth inhibition of MDA-MB-231 cell line by the *P. harmala* extract was confirmed.

**Keywords:** Cell viability; FT-IR; HPLC; Harmaline; Harmine; MTT test

1. **Background**

Herbal medicine has been used for centuries to treat many different health conditions and nowadays they are receiving increased attention, as they are cheap, locally available and have fewer side effects. A study estimated that 60 - 80% of antibacterial and anticancer drugs were derived from natural products (1).

*Peganum harmala* L. (a genera from family of Nitrariaceae), also known as Syrian Rue, is a medical herb distributed over semi arid areas of North-West India, North-Africa and Central Asia. This plant is known as “Espand” in Iran, “Harmel” in North Africa and “African Rue”, “Mexican Rue” or “Turkish Rue” in United States (2). The flowering period is March to April. The fruits are globose capsules with three chambers containing numerous small dark brown, 3 - 4 mm long seeds (3). The fruits are used as analgesic and antiseptic in folk medicine (3) and recent pharmaceutical studies proved antibacterial and antiprotozoa properties of *P. harmala* (4, 5).

It has been reported that this plant has anti-tumor effects, vasorelaxant effects, anti-HIV, anti-oxidant activity, immune-modulator properties, and hypoglycemic effects (3, 6). It has been revealed that some of the pharmacological effects of *P. harmala* may be ascribed to its β-carboline alkaloids and quinazolin derivatives (3). Alkaloids, flavonoids and anthraquinones are the main phyto-chemical compounds of *P. harmala*.

β-carboline a like harmaline, harmine, harmalol, harmol and tetrahydroharmine are the main alkaloids present in *P. harmala*. Herraiz et al. (7) identified that different parts of the plant contains different percentages of the aforementioned alkaloids for example, seeds and roots contain the highest levels of alkaloids however, stems
and leaves contain lower amounts, and flowers have no alkaloids. Herraez confirmed that harmine and harmaline accumulate in dry seeds at 4.3% and 5.6% (w/w), respectively, harmalol at 0.6%, and tetrahydroharmine at 0.1% (w/w) and roots contain harmine and harmol with 2.0% and 1.4% (w/w), respectively (7). Harmaline (dihydro-ß-carboline alkaloid) and harmine (full aromatic ß-carboline alkaloid) are the major alkaloids present in the seeds and the root of P. harmala L. (8) and inhibit monoamine oxidase A (MAO) as a main in-activator of monoaminergic neurotransmitters which is responsible for a number of neurological disorders (7, 9). Moreover, harmine showed significant tumor inhibition in mice bearing Lewis Lung Cancer, sarcoma180 or HepA tumor (10).

Cancer as one of the main public health problems around the world, is the second leading cause of death following heart disease (11). In economically developed and developing countries, breast cancer in females and lung cancer in males are the most common diagnosed cancers (12). Breast cancer is a complex and heterogeneous disease with both genetic and environmental risk factors. The incidence and mortality rates of this cancer have been rising in many African and Asian countries (12), therefore research on breast cancer is crucial to promote world health, significantly.

Breast cancer cell lines are classified based on histological type, tumor grade, lymph node status and the presence of predictive markers such as estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) (13). Breast cancer cell lines, such as MDA-MB-231, SKBR-3, MCF-12A, HBL101, MDA-MD-435, MCF-7, H559RT (14), are frequently used for basic cancer researches. The growth-inhibitory effect of Terminalia chebula fruit was confirmed on several malignant cell lines including MCF7, St15, HOS1, PC-3, and PTNIA cancer cell lines by Saleem et al. (15). Riva et al. (16) studied the anti-proliferative effects of Uncaria tomentosa extracts and its fractions on the growth of breast cancer cell line MCF7. Hostanska et al. studied the anti-proliferative activity of C. racemosa extracts (Isopropanolic and Ethanolic) on estrogen receptor positive MCF7 and estrogen receptor negative MDA-MB231 breast cancer cells by WST-1 assay (17). The positive effects of aqueous extracts of 12 Chinese medicinal herbs, Anemarrhena asphodeloides, Artemisia argyi, Commiphora myrrha, Duchesnea indica, Gleditsia sinensis, Ligustrum lucidum, Rheum palmatum, Rubia cordifolia, Salvia chinensis, Scutellaria barbata, Uncaria rhychophylla and Vaccaria segetalis were evaluated for their antiproliferative activity on eight cancer cell lines by Shoemaker et al. (18). Furthermore, alkaloids such as vinblastine, vincristine and ellipticine have been used as potent anticancer agents (19). Tumor cells were killed by these alkaloids via different mechanisms, such as induction of apoptosis and inhibition of topoisomerase I and II (20-22).

Alkaloids of P. harmala, including harmine and harmaline are effective on the human promyelocytic cell line (HL60 cells) (3). In one study, harmine significantly inhibited the growth of tumor in mice bearing Lewis Lung Cancer, sarcoma180 and HepA tumor (23). To date no study has been carried out to verify the direct human-antitumor activity of P. harmala's extracts on breast cancer MDA-MB-231 and Mcf-7 cell line.

2. Objectives

In this study, the alkaloids of P. harmala L. seeds containing herbal active principals, harmine and harmaline, were extracted and purified. Two techniques, FT-IR and HPLC, were used to for their precise identification and their effects on two cancer cell lines, MDA-MB-231 and Mcf-7 was investigated.

3. Materials and Methods

3.1. Peganum harmala’s Alkaloids Extraction

P. harmala seeds were collected from the mountains of Shahrekord, Chaharmahal Bakhhtiari province and Mashhad, Khorasan Razavi province, Iran. The grinded P. harmala seeds were mixed up with 50 mL glacial acetic acid (30% (w/w)) and were stirred for 30 minutes at low speed. The mixture was then filtered through a Buchner funnel and Whatman filter paper (No. 5) while it was washed with 20 mL glacial acetic acid (30% (w/w)) once more.

The filtrate was washed three times with petroleum ether: ethyl acetate (1:1) to remove the organic impurities by a separator funnel. The aqueous layer was collected and plenty of sodium hydroxide 10 M was added to reach a cloudy appearance. The organic part, which contained alkaloids, mainly hamaline and harmine, was captured in the chloroform phase (100 mL x three times) in a separator funnel. The solvent was finally removed by the means of rotary evaporation.

3.2. HPLC Analysis

HPLC analysis was performed using Cecil 1100 series (Cecil Inst., Ltd., Cambridge, United Kingdom) equipped with a 1100 series pump and UV absorbance detector and a column oven (CTS-30 Younglin, Korea) to detect Cyclo-maltotoadectaose (CD18), a cyclic oligosaccharides composed of 18 D-glucose units. The mobile phase consisted of potassium phosphate buffer (10 mM pH 7) and acetonitrile (50:50 v/v) with a flow rate of 1.5 mL/min at room temperature (25˚ C).

Individual stock solutions of harmine and harmaline (Sigma, USA) were prepared at five concentrations of 100 - 1000 µg.mL⁻¹ in methanol and used to draw a standard curve.

3.3. Fourier Transformation Infrared Spectroscopy (FTIR)

Fourier Transformation Infrared Spectroscopy spectra were recorded on FT-IR spectrophotometer (BRUKER,
Germany) using KBr discs. The P. harmala extracts (2 μL) were coated on the KBr discs to form thin liquid films for infrared spectrometry analysis. The discs were approximately 5 mm in diameter and 1 mm in thickness. The instrument was operated under dry air purge, and the scans were collected at scanning speed of 2 mm/s with resolution of 4 cm⁻¹ over the region of 4000 - 400 cm⁻¹.

3.4. Cell Lines

Two human breast carcinoma cell lines MDA-MB-231 and Mcf-7 were donated by Dr. Mosa Gardaneh and Eng. Azin Gholami (NIGEB). MDA-MB-231 cell line is negative for ER, PR and HER2 and positive for EGFR, Mcf-7 cell line is positive for estrogen receptor (ER) (13). Cancer cell lines were cultured in DMEM medium with 100 U.mL⁻¹ of penicillin, 100 μg.mL⁻¹ of streptomycin, and 10% fetal bovine serum (FBS). The cells were grown at 37 °C in a humidified 5% CO₂ incubator.

3.5. Chemicals

3(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), harmine (286044-1G), harmaline (51330-1G) were purchased from Sigma; DMEM, Trypsin, and FBS were purchased from Invitrogen-Life Technologies.

3.6. Cell Viability by MTT Assay

The viability of cultured cell lines was determined by the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase of viable cells. Cell lines were seeded (8000 cells/well) in a 96 well plate and incubated for 24 hours at 37 °C in 5% CO₂ incubator and different concentrations of P. harmala’s alkaloid extract (1, 10, 20, 30, 40, 50, 60, 80, and 100 μg.mL⁻¹) were added to each well and the plate was incubated for 24, 48, and 72 hours. After incubation, 20 μL MTT (5 mg.mL⁻¹) was added to each well to attain a total reaction volume of 200 μL. The plate was then kept in the incubator for a further 5 hours. Then, the medium was depleted and formazan crystals, which appeared in the last incubation step, were dissolved in 200 μL of dimethylsulfoxide. The percentage of viable cells was determined through different absorbances of analytes and controls read by ELISA plate reader at 580 nm.

3.7. Statistical Analysis

Statistical analysis was performed using the SPSS (Statistical Package for Social Sciences) software. Analysis of Variance (ANOVA) for repeated measures and Duncan’s test were performed to detect changes among the groups. Results represent means ± SD for at least three replicate determinations for the MTT test. Differences with P values < 0.001 were considered significant.

4. Results

P. harmala seeds were subjected to acidic/basic extraction to achieve their alkaloids content. Seeds and roots of P. harmala contain higher alkaloid content, in contrast leaves and stems contain the lowest amounts and flowers have no alkaloids. Harmaline and harmine are the major alkaloids present in the seeds (4.3 and 5.6% (w/w)) and the root of the P. harmala L., however, harmalol and tetrahydroharmin are present at 0.6 and 0.1%, respectively (w/w) (4). Therefore, when the extract was identified by two methods, FTIR and HPLC, two alkaloids with higher absolute concentrations were recorded, harmaline and harmine (Figures 1 and 2).

To detect the concentration of harmaline and harmine in the extract of P. harmala, the calibration curves were plotted as shown in Figure 3. The inhibitory effect of P. harmala extract on two breast cancer cell lines was tested by Methylthiazol Tetrazolium (MTT) assay. The MTT tetrazolium salt colorimetric assay measures cytotoxicity, cell proliferation and cell activation. The level of MTT cleavage by viable cells relatively addresses the increment of cell numbers during the time frame. It has been proved that the cell numbers increases non-linearly. In contrast, cell death follows another rule, a time-linear approach during MTT incubation (24).

Cancer cell lines, MDA-MB-231 and Mcf-7, were exposed to nine concentrations of P. harmala extract (1, 10, 20, 30, 40, 50, 60, 80, and 100 μg.mL⁻¹) for three days to assay the inhibitory effect of the extracts on cancer cell growth. The half maximal inhibitory concentration (IC₅₀) was used to measure the suppression of cell growth for cancer lines at different time intervals, 24, 48 and 72 hours. Therefore, it was observed that 30 μg.mL⁻¹ is the concentration at which 50% of cells die at 24 hours in MDA-MB-231 cell line (Figure 4 A). Approximately, the same concentration was recorded when cell line was exposed to harmala’s extract for longer times, 48 and 72 hours.

Furthermore, the inhibitory effect of the extract on Mcf-7 growth rate was not significant at 24 hours even in the most concentrated treatment (100 μg.mL⁻¹), which does not meet the IC₅₀ (Figure 4 B). In this cell line, it seemed that the longer exposure time has a meaningful effect on cell death. 40 μg.mL⁻¹ and 25 μg.mL⁻¹ were calculated for IC₅₀ at 48 and 72 hours, respectively. These findings confirm that both concentration and exposure time have significant effects on Mcf-7 growth inhibition through treatment with P. harmala extract.

Finally, the morphological changes of cells were studied by optical microscopy to distinguish whether apoptosis or necrosis is responsible for cell death (Figure 5). The arrows in Figure 5 indicate the round shape of the cells in both untreated and treated cell lines MDA-MB-231 and Mcf-7 with different concentrations of P. harmala extract observed under optical microscopy, 100×.
Figure 1. FT-IR Spectra for (A) Harmaline Standard and (B) Harmine Standard and (C) *P. harmala* Seeds Extract

Figure 2. HPLC Chromatogram of (A) Harmaline, (B) Harmine and (C) *P. harmala* Seeds Extract
5. Discussion

Herbs, as a natural/green resource with variety of uses including culinary and medicinal, have received much attention during all centuries. However, recently they are studied for their proper effect on some nominated diseases (15-18). Amongst them, P. harmala L., a known folklore medicine containing several alkaloids, has been proved to have anti-cancer effects (7, 20, 25). In this study, we isolated the alkaloid extraction of P. harmala seeds and identified its component by means of two analytical methods, FTIR and HPLC. Although, different alkaloids have been reported to be present in P. harmala extract i.e. harmol, harmalol, harmine and harmaline (3), we detected harmine and harmaline as the major components of the extract by FTIR. Our further analyses of cell lines determined that these two major components are the most responsible factors for such results. Figure 1 C demonstrates the absorbance of the P. harmala extract compared with harmine and harmaline standards (Figures 1 A and B) at frequency region of 4000 – 400 cm⁻¹. The spectrum of P. harmala extract was in accordance with harmine and harmaline standards and the absorptions of P. harmala extract at different wave lengths, 1072, 1237, 1455, 1624 and 3072 refers to different functional groups (C-H), (C=O), (C=N), (OCH₃) and (C-N), respectively.

HPLC analysis was used to separate the P. harmala extract components and compared the results with the standard solutions of harmine and harmaline. The chromatograms approved the presence of harmine and harmaline as their retention time at 8.07 and 5.32 minutes, respectively were in accordance with the standard solution (Figures 2 A, B, and C). Further analysis of HPLC indicated that harmaline has higher concentration in the P. harmala extract. By means of plotting calibration curves, concentration of 640 μg.mL⁻¹ and 189 µg.mL⁻¹ for harmaline and harmine, respectively were indicated.

It has been reported that harmine and harmaline have similar pharmaceutically equivalent affects; however, harmine is supposed to be less poisonous, therefore it has more advantageous for use in cancer therapy. In the present research we used total P. harmala alkaloids extract to show its effectiveness against growth of two breast cancer cell lines, in vitro. The results of proliferation inhibitory rate analysis (Figures 4 A and B) confirmed that the more concentrated extract, the more potential in cell growth inhibition. However, time of exposure, as another parameter plays an important role in increasing efficiency of cell growth suppression. Although we had to conclude the first parameter, concentration, is a function of
Figure 5. Cell Morphology of MDA-MB-231; the First Row, (A) Untreated MDA-MB-231 (B) Treated MDA-MB-231 with 25 μg.mL⁻¹ of P. harmala Extract for 24 Hours (C) Treated MDA-MB-231 with 100 μg.mL⁻¹ of P. harmala Extract for 24 Hours. Cell Morphology of Mcf-7; the Second Row, (D) Untreated Mcf-7 (E) Treated Mcf-7 With 40 μg.mL⁻¹ of P. harmala Extract for 48 Hours (F) Treated Mcf-7 With 100 μg.mL⁻¹ of P. harmala Extract for 48 Hours (optical microscopy, 100x).

The arrows show the round-shape of cells that confirms apoptosis is responsible for cell death.

other parameter exposure time; since time of exposure did not provide a significant difference when treating MDA-MB-231 with P. harmala extract as the suppression of cell growth for cancer line MDA-MB-231 occurred in lesser time (24 hours) compared with Mcf-7. In contrast, exposure time seems to be crucial in case of Mcf-7 treatment with P. harmala extract; precisely, longer exposure time decreased cell growth rate more vigorously especially at 72 hours. Considering 30 - 40 μg.mL⁻¹ of the extract as IC₅₀, we can conclude that concentration of the extract has greater importance than time of exposure to provide higher cytotoxic effect and cell death. Besides, it is worth noting that MDA-MB-231 cell line naturally has a higher growth rate than Mcf-7 cell line, which may explain higher growth inhibition of MDA-MB-231 cell line by the P. harmala extract. Other studies regarding breast cancer cell line treatment took advantage of hormone replacement therapy. Hostanska et al. found that proliferative activity and cell killing occurs when they exposed MCF-7 and MDA-MB231 against isopropanolic and ethanolic extracts of Cimicifuga racemosa (17). The effective dose for IC₅₀ was calculated to be 80.6 ± 17.7 μg.mL⁻¹ in MCF-7 cells and 58.6 ± 12.6 μg.mL⁻¹ in MDA-MB231 cells when an ethanolic extract was used. Their results confirmed apoptosis mode of cell death according to microscopic inspection and further analysis.

Furthermore, morphological changes in cell shape investigated under optical microscopy revealed that the lethal effect of P. harmala extract on cancer cell line could be a kind of cell death or apoptosis, as no membrane permeabilization was observed and cells kept their membrane intact. However, we believe that other apoptotic
parameters at the molecular level (caspase activation, cytchrome c release, and oligonucleosomal DNA fragmentation) should be followed to confirm this observation. Similar results by Zhao and Wink, indicate that harmine induces senescence processes in cells which leads to expedited cell death (26). Furthermore, our diagnosis of apoptotic cell death is supported by Hostanska et al. (17).

In conclusion, the results of the current research address the anti-cancer effect of P. harmala L. to its alkaloid components mainly harmine and harmaline. The P. harmala extract exposure against two cancer cell lines, MDA-MB-231 and MCF-7, showed cell growth inhibition and in higher concentration/longer time, complete cell death occurred. Cell mortality rate and IC₅₀ data confirmed dose/time-dependent inhibition effect of P. harmala on this cancer cell line.

Further studies are suggested to elucidate the mechanism of action of both harmine and harmaline on more human cancer cell lines and eventual use of these herbal active principle compounds in future anti-cancer pharmaceuticals should be considered.

Acknowledgements
There is no acknowledgment.

Authors' Contribution
All authors have participated equally in the present study.

Financial Disclosure
The authors declare that they have no conflict of interest.

Funding/Support
This work was financially supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB).

References