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Original Article

Efficiency of ß-carbolines Presented in the Seeds of *Peganum harmala* L. as Antiproliferative Agent Against Breast Cancer Cell Line

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ABSTRACT

Harmaline and harmine, are the major ß-carbolines present in the seeds of the *Peganum harmala* L. These compounds are known as herbal active principals with potential use in pharmaceutical and medicine. To assess the growth inhibitory effect of phyto-alkaloids, *harmaline* and *harmine*, on cancer cell lines. The *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). breast cancer cell lines, MDA_MB_231, were subjected to different concentration (1–100 µg/mL) of the *P. harmala* extract at different time courses (24h, 48h). Methylthiazol Tetrazolium (MTT) test, the half maximal inhibitory concentration (IC_{50}) and the morphological changes through optical microscopy were evaluated 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 24h.It was observed that 30 µg/mL is the minimum lethal dose that kills approximately 50% of cells at 24 hours in MDA-MB-231 cell line (IC_{50}). The morphological observation ensured the apoptosis nature of *P. harmala* on cells as their membrane kept intact and no membrane permeabilization was observed. The results revealed that the *P. harmala* extracts decreased significantly growth rate and cell survival of cancer cell lines. higher growth inhibition of MDA-MB-231 cell line by the *P. harmala* extract was confirmed.

INTRODUCTION

Herbal medicine has been used for centuries to treat many different health conditions and now a day they are receiving increased attention as they are cheap, locally study estimated that 60-80% of antibacterial and anticancer drugs were derived from natural products [1]. *Peganum harmala* L. (a genus from family of *Nitrariaceae*), also known as Syrian Rue, is a medical herb distributed over semiarid 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 the United States [2]. The flowering period is March to April. The fruits are globose capsules with 3 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 anti-protozoa of *P. harmala* [4,5].

It has been reported that this plant provides 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 by its β-carboline alkaloids and quinazolin derivatives [3]. Alkaloids, flavonoids, and anthraquinones are the main phyto-chemical compounds from *P. harmala*.

β-carboline a like *harmaline*, *harmine*, *harmalol*, harmol, and tetrahydro*harmine* are the main alkaloids presenting in *P. harmala*. Herraiz *et al.* (2010) identified that different parts of the plant contain a different percentage of the aforementioned alkaloids, for example, seeds and roots containing the highest levels of alkaloids however, stems and leaves contain a lower amount, and flowers have no

alkaloids. Herraiz confirmed that *harmine* and *harmaline* accumulated in dry seeds at 4.3% and 5.6% (w/w), respectively, *harmalol* at 0.6%, and tetrahydro*harmine* at 0.1% (w/w) and roots contained *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 presenting in the seeds the *P. harmala* L. [8], 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 in 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 studying about breast cancer is crucial to promote world health, significantly.

The 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, HS598T [14], are frequently used for basic cancer researches. The growth-inhibitory effect of *Terminalia chebula* fruit was confirmed on several malignant cell lines including S115, HOS-1, PC-3, and PNT1A cancer cell lines by Saleem *et al*. (2002) [15]. Riva *et al*. (2001) has studied the antiproliferative effects of *Uncaria tomentosa* extracts and its fractions on the growth of breast cancer cell line MCF7 [16]. Hostanska *et al*. (2004) has studied the antiproliferative activity of *C. racemosa* extracts (isopropanolic and ethanolic) on the 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*. (2005) [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 mechanism, such as induction of apoptosis, 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 a study, *harmine* inhibit the growth of tumor in mice bearing Lewis Lung Cancer, sarcoma180 and HepA tumor significantly [23]. To date, no studies have been carried out to verify the direct human-antitumor activity of the *P. harmala*'s extracts on breast cancer Mcf-7 cell line. In this study the alkaloids of *P. harmala* L. seeds containing herbal active principals, *harmine* and *harmaline*, were extracted and purified and detected by FT-IR and HPLC and its antiproliferative effects on tumor cell lines, MDA-MB-231, was studied.

MATERIALS AND METHODS

Peganum Harmala's Alkaloids Extraction

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

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

HPLC Analysis

The HPLC analysis was performed using Cecil 1100 series (Cecil Inst., Ltd., Cambridge, United Kingdom) equipped with an 1100 series pump and UV absorbance detector and a column oven (CTS-

30 Younglin, Korea) to detect Cyclomaltooctadecaose (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 flow-rate 1.5 ml/min at temperature (25 °C).
Individual stock solutions of *harmine* and *harmaline* (Sigma, USA) were prepared at five concentrations of 100-1000 µg/mL in methanol and used to draw standard curve.

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 scans were collected at scanning speed of 2 mm/s with resolution of 4 /cm over the region of 4000–400 /cm.

Cell Lines

The human breast Carcinoma cell line MDA-MB-231 were donated by (NIGEB), is negative for ER, PR and HER2 and positive for EGFR, [13]. Cancer cell line was cultured in DMEM medium with 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal bovine serum (FBS). The cell was grown at 37 °C in a humidified 5% CO_2 incubator.

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.

Cell Viability by MTT Assay

The viability of cultured cell lines was determined by 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 96 well plate and incubated for 24 h at 37 °C in 5% CO_2 incubator then, different concentrations of *P. harmala*'s alkaloid extract (1, 10, 20, 30, 40, 50, 60, 80 and 100 µg/mL) were added to each well and the plate was incubated for 24, 48. After incubation, 20 µL MTT (5 mg/mL) was added to each well to attain a total reaction volume of 200 µL. The plate was then kept in the incubator for further 5 h. 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 the different absorbance of analytes and controls read by ELISA plate reader at 580 nm.

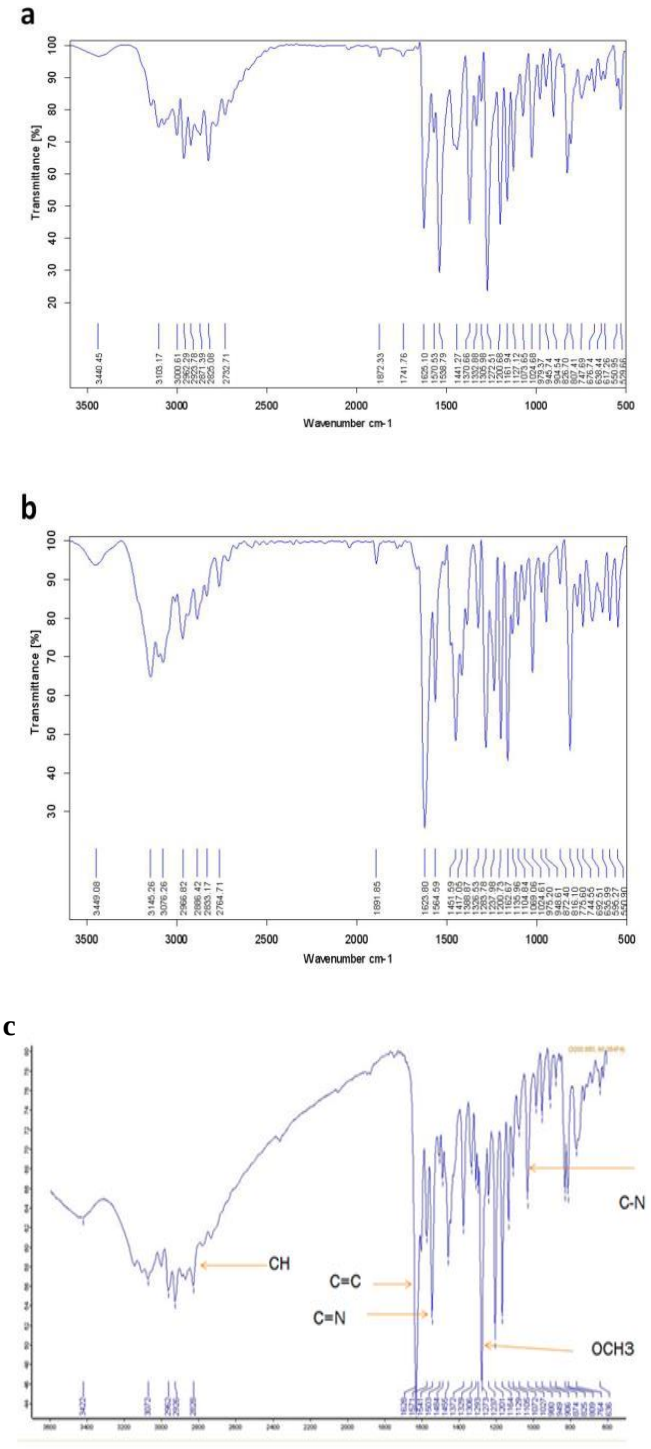

Fig. 1 FT-IR spectra in the region of 4000 – 500/cm for (a) Harmaline standard, (b) Harmine standard and (c) *P. harmala* seed extract

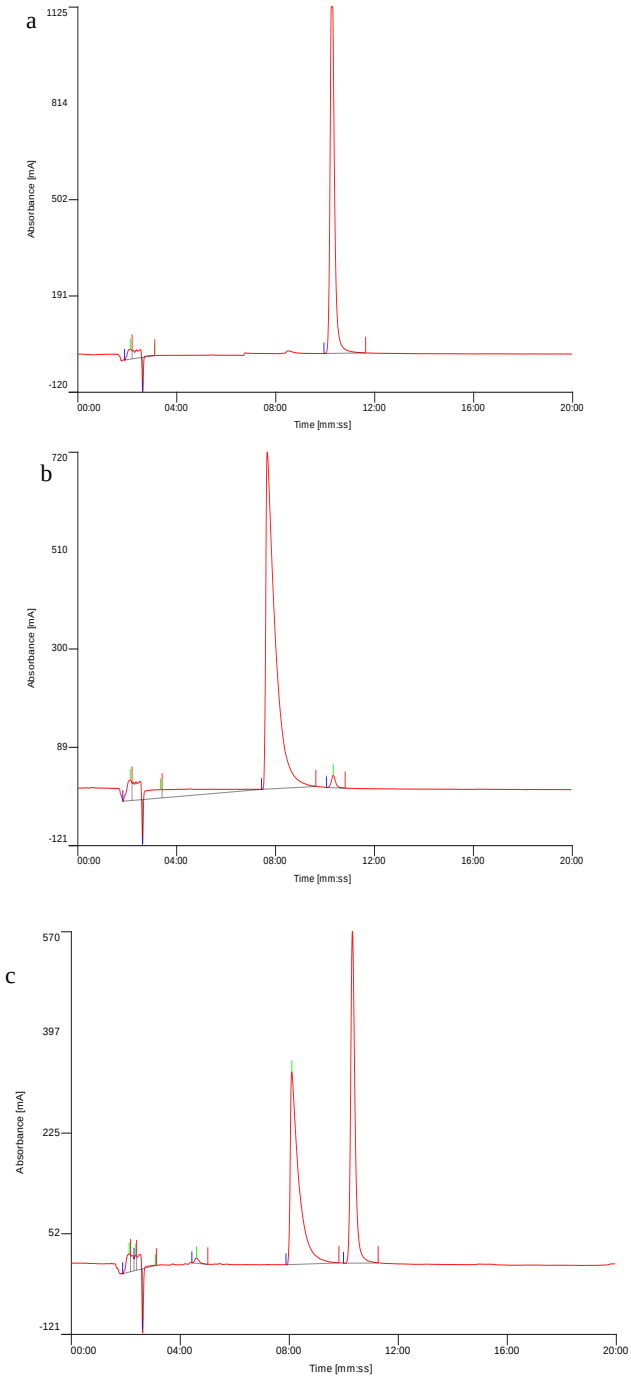

Fig. 2 HPLC chromatogram of (a) *Harmaline* and (b) *Harmine* and (c) *P. harmala* seeds extract Chromatogram of peganum harmala seed extract: harmine (8.3) and harmaline (10.89) according to

Statistical Analysis

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

RESULTS

P. harmala seeds were subjected to acidic/basic extraction to achieve their alkaloids content. Seeds of *P. harmala* contain higher alkaloids content, *Harmaline* and *harmine* are the major alkaloids presenting in the seeds (4.3 and 5.6 % (w/w) [4]. Therefore, when the extract is identifying by two methods, FTIR (Fig. 1) and HPLC, we are recording two alkaloids, h*armaline* and *harmine*, for their absolute higher concentration (Fig. 2).

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. It has been approved that the cell numbers increase non-linear. In contrast, the cell death follows another rule, a time-linear approach during MTT incubation [24].

Cancer cell lines, MDA-MB-231, was exposed to nine concentrations of *P. harmala* extract (1, 10, 20, 30, 40, 50, 60, 80, and 100 µg/mL) for three days to assay the inhibitory effect of the extracts on cancer cell growth. The half maximal inhibitory concentration (IC_{50}) used to measure the suppression of cell growth for cancer lines cancer lines at different time intervals 24h, 48. Therefore, it was observed that 30µg/mL is the conc. where 50% of cells die at 24 hours in MDA-MB-231 cell line

The same concentration was calculated for longer exposure time, 48 h, approximately (Fig. 3).

Finally, the morphological changes of cells were studied by optical microscopy to distinguish whether apoptosis or necrosis is responsible for cell death (Fig. 4).

The round shape of the cells in both untreated and treated cell lines MDA-MB-231 with different concentrations of *P. harmala* extract observed under optical microscopy, 100X.

DISCUSSION

Herbs, as a natural/green resource with a variety of uses including culinary and medicinal, have received a lot of attention in all centuries. But recently they are studied for their proper effect on some nominated diseases [15-18]. Of them, *P. harmala* L., a known folklore medicine containing several alkaloids, have used to prove its anti-cancer effect [7,25,26].

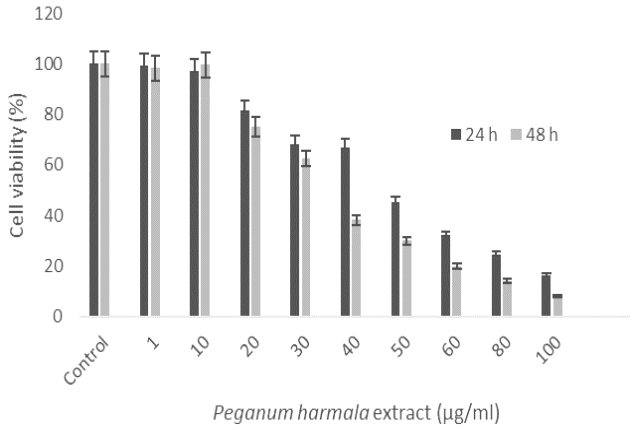

Fig. 3 Inhibition of proliferation of MDA-MB-231 cancer cells by means of MTT assay. Each value represents the mean ± standard devation of more than three replicates. $p < 0.001$

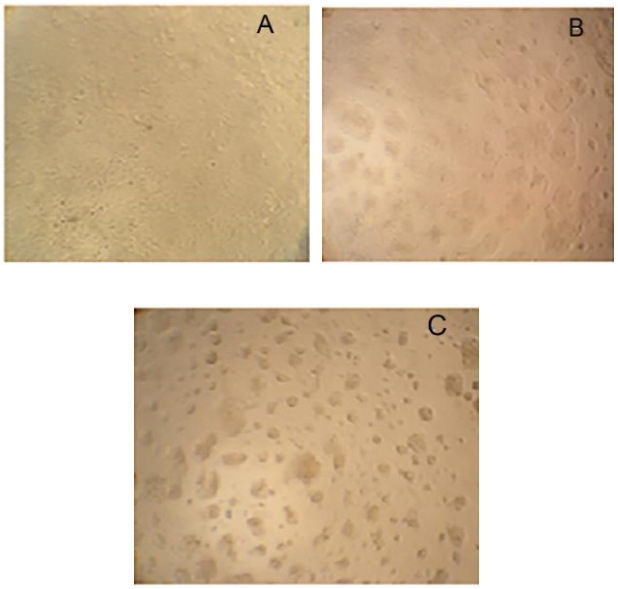

Fig. 4 Cell morphology of MDA-MB-231, (A) un treated MDA-MB-231 (B) treated MDA-MB-231with 25 µg/mL of *P. harmal* extract in 24h (C) treated MDA-MB-231with 100 µg/mL of *P. harmal* extract in 24h. (Optical microscopy, 100X).

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 are reported to be presented in *P. harmala* extract i.e. *harmol*, *harmalol*, *harmine* and *harmaline* [3], we detected *harmine* and *harmaline* as the major component of the extract by FTIR. Our further analyses with cell lines address these two major components as the most responsible factors for such results. Figure 1C demonstrates the absorbance of the *P. harmala* extract compared with *harmine* and *harmaline* standards (Fig. 1 A and B) at frequency range of 400–4000 /cm. The spectrum of *P. harmala* extract was in accordance with *harmine* and *harmaline* standards and the absorptions of *P. harmala* extract at different wavenumbers, 1072, 1237, 1455, 1624 and 3072 referred to different functional groups (C-H), (C=O), (C=N), (OCH3), (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 min, respectively were according to the standard solution (Fig. 2A, B, and C).

Further analysis of HPLC indicated that *harmaline* has a higher concentration in the *P. harmala* extract. By means of plotting calibration curves, concentrations of 640 µg/mL and 189 µg/mL for *harmaline* and *harmine* were indicated, respectively. It is reported that *harmine* and *harmaline* have similar pharmaceutically equivalent action; however, *harmine* is supposed to be less poison; therefore, it has more advantageous to be used in cancer therapy. In the present research, we used total *P. harmala* alkaloids extract to show its effectiveness against the growth of breast cancer cell line, *in vitro*. The results of proliferation inhibitory rate analysis (Fig. 3A and B) confirmed that the more concentrated extract we used, the more potential in cell growth inhibitory reached. However, time of exposure, as another parameter plays an important role in increasing the efficiency of cell growth suppression. Although we had to conclude this parameter as a function of other parameters, since the 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 (24h). Considering 30-40 µg/mL of the extract as IC_{50}, it is to conclude that concentration of the extract has greater importance than time of exposure to provide higher cytotoxic effect and cell death. Other studies regarding breast cancer cell line treatment took advantage of hormone replacement therapy. Hostanska *et al.* 2004 found that proliferative activity and cell killing occurs when they exposed MDA-MB231 against isopropanolic and ethanolic extracts of *Cimicifuga racemosa* [17]. The effective dose for IC_{50} has been calculated 58.6±12.6 µg/mL µg/mL in MDA-MB231 cells when ethanolic extract was used. Their results confirmed the apoptosis

mode of cell death according microscopic inspection and further analysis.

Even more, the morphological changes in cell shape investigated under optical microscopy revealed the lethal effect of *P. harmala* extract on cancer cell line could be a kind of cell death, apoptosis, as no membrane permeabilization was observed and cells kept their membrane intact.

However, we believe other apoptotic parameters at molecular level (*caspase* activation, cytochrome c release, and oligonucleosomal DNA fragmentation) should be followed to confirm this observation. The similar results by Zhao and Wink, 2013 indicate that *harmine* induces senescence process in cell which leads expedited cell death [27]. Furthermore, our diagnosis on the apoptosis cell death is supported by Hostanska *et al*. 2004 [17], as well.

In conclusion, the results of the current research address the anti-cancer effect of *P. harmala* L. to its alkaloid components mainly *hamine* and *harmaline*. The *P. harmala* extract exposure against cancer cell line, MDA-MB-231 showed cell growth inhibition and in higher concentration/longer time, complete cell death was occurred. Cell mortality rate and IC_{50} data confirmed dose/time-dependent inhibition effect of *P. harmala* on this cancer cell line.

It is suggested to perform further studies to elucidate the mechanism of action of both *harmine* and *harmaline* on more human cancer cell lines and eventual use of these herbal active principal compounds in future anti-cancer pharmaceuticals is considerable.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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