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Research paper

Evaluation of the cytotoxic and cytostatic activities of alkaloid extracts from different parts of *Peganum harmala* L. (Zygophyllaceae)

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ABSTRACT

Introduction: *Peganum harmala* L. (*P. harmala*) is broadly used in folk medicine for the treatment of various diseases including cancer. The seeds are particularly employed for therapeutic. The present paper investigates the cytotoxic effects of raw alkaloid extracts from different parts of *P. harmala* including fruits (TAFr), seeds (TASe), roots (TAR) and aerial parts (TAAp); in order to assess traditional claims about the therapeutic potential of this plant.
Methods: The cytotoxic effects were evaluated on six malignant cancer cells (A549, U373, Hs683, MCF7, B16F10 and SKMEL-28; A549, U373, MCF7 and SKMEL-28 are resistant to proapoptotic stimuli) by MTT assay and quantitative videomicroscopy analysis. The main alkaloids were quantified by HPLC in the different parts of plant.
Results: Total alkaloids of the different parts were cytotoxic towards practically all cancer cell lines with IC_{50} ranging 1–52 μg/mL after 72 h of treatment. Videomicroscopy analysis indicated that the TAFr, TASe and TAR alkaloids affect A549 lung carcinoma cells behaviour and induce a cytostatic effect whereas TAAp extract was cytotoxic rather than cytostatic. TAAp, TAFr, TASe and TAR treatment induced global growth ratio indexes (GGR) of 0.19, 0.26, 0.3 and 0.62, respectively, after 72 h of treatment. Depending on the organe, harmine, harmaline, harmol and vasicine have, range between 2 and 90% w/w of total alkaloids.
Conclusion: These data indicate that *P. harmala* alkaloids extract may support the traditional claims regarding its anticancer uses which could be helpful in providing of new cytotoxic agents against chemo-resistant cancer cells.

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1. Introduction

Cancer remains the second leading cause of death behind cardiovascular diseases; cancer-related deaths are predicted to increase to over 24 million in 2035 [1,2]. The use of cancer chemotherapy began at the start of the 20th century and, for the time being, small molecules remain the principal mode of treatment for various cancers [3]. A total of 174 new anticancer drugs were approved during the time period 1981–2014 by the FDA (food and drug administration) [4]. Chemotherapy drugs can potentially eliminate cancer cells but can also damage perfectly healthy cells and tissues; these induce side effects throughout the body, dramatically reducing the quality of life for patients [5]. Hence, there is a requirement to discover new potent anticancer drugs with fewer side effects. Based on past major successes, medicinal plants are considered a promising source to develop new chemical compounds with potential anticancer activities [6].

P. harmala belongs to the family of Zygophyllaceae. Widely distributed in North Africa, it grows naturally in semi-arid and pre-desert area. It has wide uses in folk medicine for the treatment of various diseases; it has anti-inflammatory [7], anticancer [8], analgesic, antiseptic [9], and stimulant emmenagogue [10]

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properties and is traditionally used to treat diabetes, hypertension [11], lumbago, asthma, colic and jaundice [10]. Several studies have also described therapeutic effects of *P. harmala*, such as antibacterial [12], antiplasmodial, vasorelaxant [13,14], hypoglycemic [15] and phytotoxic effects [16]. The antitumor effects of *P. harmala* seeds on cancerous cell lines have been demonstrated in previous studies but mechanisms of action are still unknown [2,8,17–19]. Some studies also reported the cytotoxic effects of harmine derivatives [20,21].

To the best of our knowledge, there are no publications focusing on the cytotoxic activity of alkaloid extracts obtained from different parts (seeds, fruits, roots and aerial parts) of *P. harmala*. In this study, we evaluate for the first time the cytotoxic effects of total alkaloid extracts on six cancer cell lines using MTT assay. In addition, the changes in cell proliferation, cell death and cell morphology induced by tested extracts were investigated by means of computer-assisted phase-contrast microscopy (quantitative videomicroscopy analysis).

2. Material and methods

2.1. Plant extraction

The different parts of *P. harmala* (aerial parts, fruits, seeds and roots) were collected in the highlands of Sétif (Algeria) in June 2011. A voucher specimen was deposited at the Herbarium of the Botanical Garden of Meise, Belgium (BR0000013496968). The extraction was carried out according to the method described by Kartal et al. (2003) with some modifications [22]. Dried plant materials (20 g) were crushed and then extracted with 200 mL ethanol (80%, v/v) in a soxhlet apparatus. The ethanol extracts were filtered and concentrated under vacuum, acidified with HCl (2%, v/v) and extracted with petroleum ether to remove fatty materials. The aqueous layer was brought to pH 9 with ammonia and extracted three times with chloroform (20 mL). The organic layer was evaporated under vacuum to obtain total alkaloids from aerial parts (TAAp), fruits (TAFr), seeds (ATSe) and roots (ATR).

2.2. Quantification of alkaloids

In the different extracts, the major alkaloids were quantified by HPLC method. The chromatographic separation was performed on a HPLC Kontron System. The compounds were eluted on a Luna 5 μm phenyl hexyl column (250 × 4.60 mm) (Phenomenex, Utrecht, Netherlands) using a gradient of 0.1% formic acid in water (phase A) and $CH_3CN—CH_3OH$ (1:1, v/v; phase B) at a flow rate of 1.5 mL/min at 40 °C. The gradient was as follows: equilibration time, 15 min at 0% B; 0 min, 0% B; 5 min, 10% B; 10 min, 25% B; 15 min, 50% B; 17 min, 100% B. The UV-detector was set at 254 nm and 20 μL samples were injected [7].

2.3. Cytotoxic activity

2.3.1. Preparation of test solutions

The stock solutions of *P. harmala* alkaloid extracts were prepared in DMSO (10 mg/mL) and diluted with the culture media to achieve the indicated final concentrations (0.5–100 μg/mL; maximum 1% DMSO in contact with cells). The etoposide used as positive control was purchased from Sigma (Saint Louis, Missouri, USA). The stock solution of etoposide was prepared in DMSO (10^{-2} M) and diluted with the culture media at desired concentration.

2.3.2. Cell culture

Human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and the Deutsche

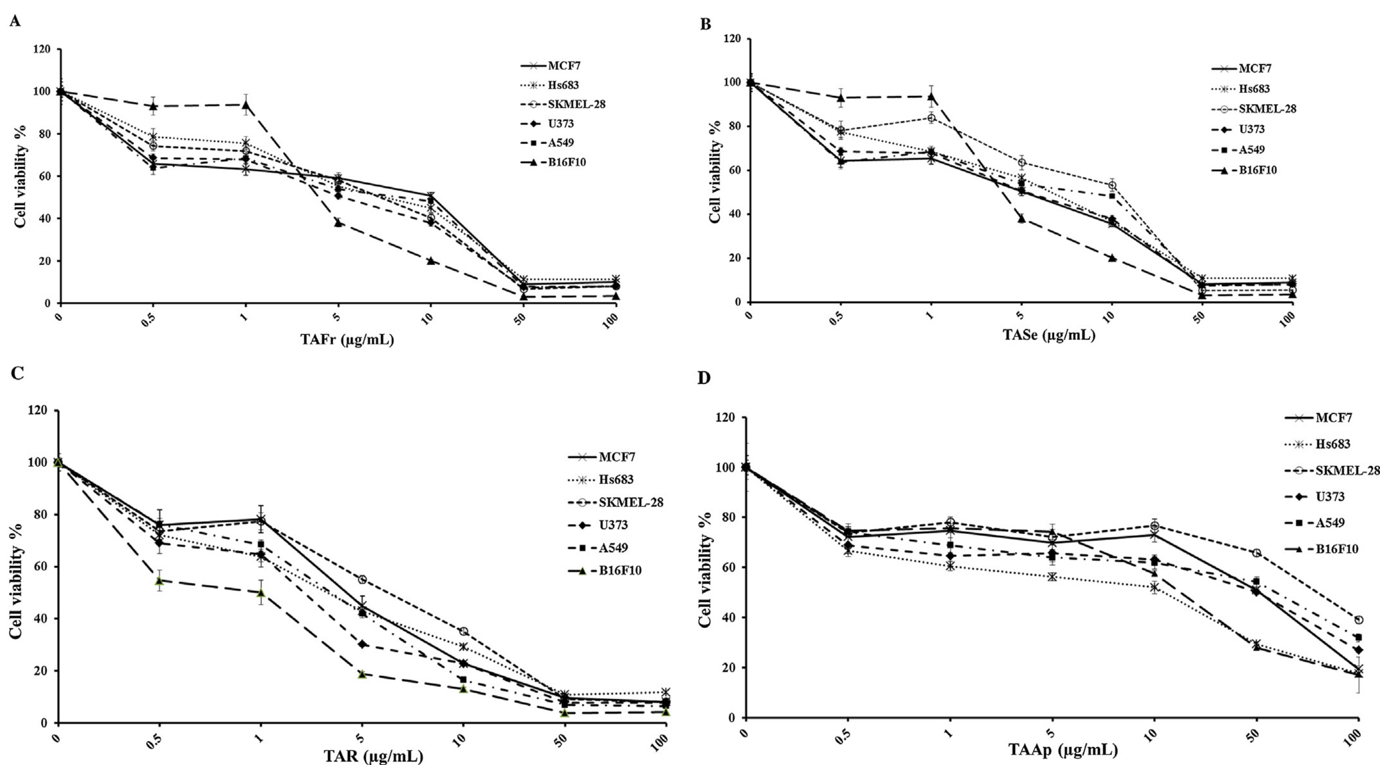

Fig. 1. *P. harmala* alkaloid extracts inhibited the viability of six malignant cancer cells (MCF7, Hs683, SKMEL-28, U373, A549 and B16F10) in a dose-dependent manner. Cells were treated with DMSO vehicle or the indicated concentrations of TAFr (A), TASe (B), TAR (C), and TAAp extracts (D) for 72 h. Cell viability was determined using MTT assay and expressed as means ± SEM in three independent experiments. TAFr: total alkaloid of fruits; TASe: total alkaloid of seeds; TAR: total alkaloid of roots; TAAp: total alkaloid of aerial parts.

Table 1
IC_{50} values (μg/mL) of total alkaloids from different parts of *P. harmala* against various cancer cell lines after 72 h incubation. IC_{50} values were determined based on the dose-response curves shown in Fig. 1 (means ± SEM of three separate experiments).

Total alkaloid extract	IC_{50} (μg/mL)					
	MCF7	Hs683	SKMEL-28	U373	A549	B16F10
TAAp[a]	15.5 ± 4.1	52.7 ± 7.0	80.2 ± 3.7	46.6 ± 5.5	55.5 ± 7.5	20.0 ± 0.8
TAFr	8.1 ± 1.4	12.1 ± 2.6	7.9 ± 1.7	15.6 ± 1.6	18.1 ± 1.2	3.4 ± 0.4
TASe	6.6 ± 0.5	4.4 ± 0.8	11.7 ± 1.4	5.4 ± 0.3	8.7 ± 1.5	4.1 ± 0.2
TAR	3.7 ± 0.4	4.6 ± 0.5	6.2 ± 0.5	2.6 ± 0.3	3.8 ± 0.4	1.0 ± 0.1
Etoposide[b]	3.5 ± 0.6	25.5 ± 0.9	4.2 ± 0.3	48.0 ± 1.1	0.9 ± 0.03	n.d.[c]

[a] TAAp: total alkaloid of aerial parts; TAFr: total alkaloid of fruits; TASe: total alkaloid of seeds; TAR: total alkaloid of roots.
[b] the growth inhibitory effects of etoposide are expressed as μM.
[c] not determined.

Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Six cell lines were used to evaluate the cytotoxic activity: human A549 non-small-cell-lung cancer (NSCLC) (DSMZ code ACC107), human U373 glioblastoma (ATCC code HTB-17), human Hs683 anaplastic oligodendroglioma (ATCC code HTB-138), human MCF7 breast cancer (ATCC code HTB-22), human SKMEL-28 melanoma (ATCC code HTB-72) and mouse B16F10 melanoma (ATCC code CRL-6475). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 μg/mL gentamicin, and penicillin-streptomycin (200 U/mL and 200 μg/mL) 24 h before the start of the experiment. Cells were grown at 37 °C in a humidified 5% CO_2 incubator.

2.3.3. MTT assay

The overall growth level of human cancer cell lines was determined using the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]diphenyl tetrazoliumbromide, Sigma) assay [23]. Briefly, the cell

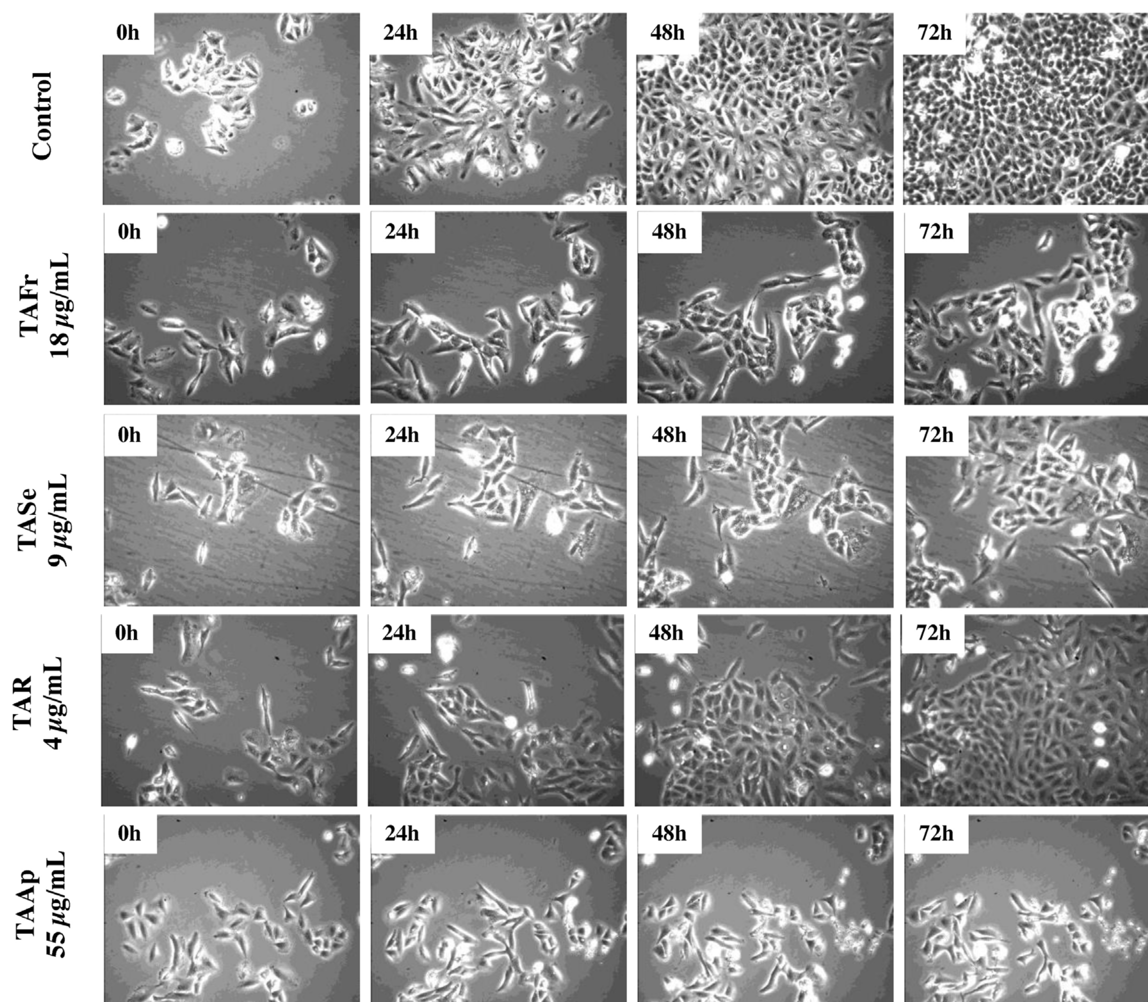

Fig. 2. Morphological illustrations of A549 human lung carcinoma cell populations untreated (control) or treated for 72 h with respective *in vitro* growth inhibitory IC_{50} value of TAFr, TASe, TAR and TAAp, as determined using the MTT assay in A549 lung carcinoma (see Table 1). TAFr: total alkaloid of fruits; TASe: total alkaloid of seeds; TAR: total alkaloid of roots; TAAp: total alkaloid of aerial parts.

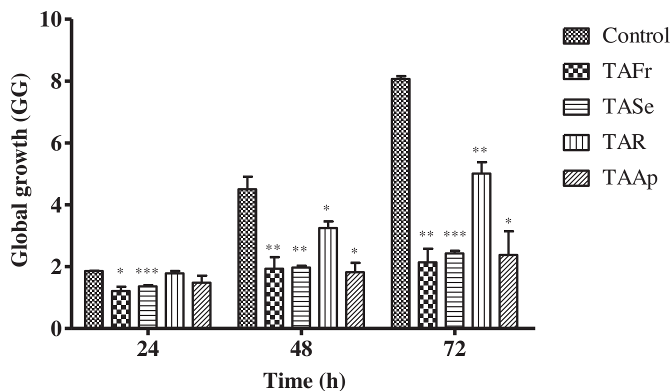

Fig. 3. Effect of IC_{50} treatment on the global growth of A549 human lung cancer cells. The data are expressed as means ± S.E.M of three separate experiments (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001 compared with control group. TAFr: total alkaloid of fruits; TASe: total alkaloid of seeds; TAR: total alkaloid of roots; TAAp: total alkaloid of aerial parts.

lines were incubated for 24 h in 96-microwell plates (at a density of 5000–8000 cells/well). The assessment of cell population growth by means of the MTT colorimetric assay is based on the capability of living cells to reduce the yellow product MTT to a purple formazan by a reduction reaction occurring in the mitochondria. As incubation over 1 cell cycle (~24 h) does not allow to reveal all possible events affecting cell viability, cytotoxicity studies duration was fixed at 72 h. The number of metabolically active *("living")* cells after 72 h of culture in the presence (or absence: control) of the alkaloid extracts is directly proportional to the intensity of the purple formazan product measured quantitatively by spectrophotometry (Biorad model 680XR, Biorad, Nazareth, Belgium) at 570 nm wavelength (with a reference of 630 nm). The measured absorbance directly correlates to the number of viable cells. The percentage of cell survival was calculated as follows: % cell survival = (absorbance of treated cells/absorbance of cells with vehicle solvent) × 100. The half inhibitory concentration (IC_{50}) was calculated from the dose-response curve obtained by plotting the percentage of cell survival versus the concentration of plant extract used. Each set of experimental conditions was carried out in triplicates [24].

2.3.4. Computer-assisted phase-contrast microscopy (quantitative videomicroscopy)

The direct visualization of A549 lung carcinoma behaviour after treatment with the alkaloid extracts under study, *i.e.* inhibition of cell proliferation (a cytostatic effect) versus direct induction of cell death (a cytotoxic effect), was carried out by means of computer-assisted phase contrast microscopy, *i.e.* quantitative videomicroscopy, as previously detailed [25]. Briefly, an image of the seeded flask bottom was acquired every 4 min over the course of a 72 h observation period. Thus, 1080 digitized images were available for each experimental condition and an approximately 40 s movies were generated from these images. Based on the phase-contrast pictures of each analyzed extract, a global growth ratio index (GGR index) was calculated [25] and compared to the MTT IC_{50} value. The global growth (GG) is calculated in each control and in each treated condition by dividing the number of cells on the last image by the number of cells on the first image. The GGR index is obtained for each extract by dividing the GG values calculated for treated A549 cells by the GG values calculated for the control.

2.4. Statistical analysis

Results are expressed as mean ± S.E.M. of at least three independent experiments. Student's *t*-test was used to compare the difference between each treated group and the control. *P*-values of less than 0.05 were considered significant.

3. Results

3.1. Determination of the IC_{50} of P. harmala *alkaloid extracts on cancer cell lines*

The MTT assay is a method generally used to study the action of natural compounds and plant extracts on cell proliferation, viability and cytotoxicity, as described by Mosmann, 1983 [26]. In this experiment, we determined the IC_{50} *in vitro* growth inhibitory activities of four extracts of *P. harmala* under study, at concentrations of up to 100 μg/mL. These extracts exhibit a marked growth inhibitory activity toward all the cancer cell lines tested, regardless of whether the cancer cells are resistant (A549, U373, MCF7 and SKMEL-28 models) or sensitive (Hs683 and B16F10 models) to proapoptotic stimuli (Fig. 1) with IC_{50s} ranging 1–52 μg/mL (Table 1). However, the viability of SKMEL-28 cells

Table 2
Quantitative videomicroscopy data.

Alkaloid extracts		Global growth ratio (GGR index)[a]	Global profile of the compound in terms of growth inhibition	% Cell death (±SEM)
TAFr[b]	24 h	0.65	Cytostatic	0.7 ± 0.7
	48 h	0.43		1.6 ± 0.3
	72 h	0.26		1.3 ± 0.2
TASe	24 h	0.73	Cytostatic	1.4 ± 0.7
	48 h	0.44		2.8 ± 0.9
	72 h	0.30		2.1 ± 0.6
TAR	24 h	0.96	Cytostatic	1.0 ± 1.0
	48 h	0.72		2.0 ± 1.2
	72 h	0.62		1.8 ± 0.7
TAAp	24 h	0.64	Cytotoxic	4.7 ± 3.8
	48 h	0.31		19.5 ± 12.7
	72 h	0.19		27.9 ± 19.6

[a] global growth ratio index (GGR index) determined by quantitative videomicroscopy on A549 lung cancer cell line at the MTT colorimetric assay related IC_{50} concentration of each alkaloid extract.
[b] TAFr: total alkaloid of fruits; TASe: total alkaloid of seeds; TAR: total alkaloid of roots; TAAp: total alkaloid of aerial parts.

was only slightly decreased after treatment with TAAp at 80 μg/mL; besides, up to 38% of SKMEL-28 cell survived after treatment with 100 μg/mL (the highest tested dose). All total alkaloid extracts exhibited a potent inhibition on the viability of mammalian cancer cells, MCF7 (IC_{50} < 15 μg/mL) (Table 1). The TAFr, TASe and TAR exhibited a significant reduction in the cell viability with a low IC_{50} in comparison with control (etoposide). Especially, TAR displayed a markedly decreased on cancer cells growth (IC_{50} < 6 μg/mL) (Table 1).

3.2. Cytotoxic and cytostatic effects of different alkaloid extracts of P. harmala on A549 lung cancer cells

In order to determine whether the plant extracts induced cytotoxic or cytostatic effects, we submitted the human A549 cell line to quantitative videomicroscopy analysis (computer-assisted phase-contrast microscopy). The morphological data (Figs. 2 and 3) indicate that the TAFr, TASe and TAR displayed cytostatic and not cytotoxic effects on the A549 cells, which are resistant to various proapoptotic stimuli (percentage of cell death was very low <2.8%); by contrast, the TAAp displayed cytotoxic rather than cytostatic effects (Table 2). Consequently, the GGR value of the alkaloid extracts from different parts of *P. harmala* decreased in a time-dependent manner (Table 2).

The TAAp, TASe and TAFr displayed a GGR value of 0.19 (means that 19% of cells growth) and 0.26 (26% of cells growth), respectively after 72 h of treatment. However, a GGR value of 0.62 (62% of cells growth) was obtained for the TAR (Table 2). The percentages of cell death, calculated for each alkaloid extracts, indicate that, when assayed at their MTT IC_{50s}, TAFr, TASe and TAR inhibit cancer cell proliferation without inducing cell death (Fig. 4) in the A549 NSCLC cell line. By contrast, the TAAp extract led to 29% cell death in the same cells (Table 2).

3.3. Alkaloids quantification

In the different parts of *P. harmala*, the major quinazoline and β-carboline alkaloids were quantified by HPLC. A R^2 value typically higher than 0.98 was observed for linear fitting of the curves obtained using standards prepared in 0.1% HCOOH: harmine and harmol (10–80 mg/mL), harmaline (25–250 mg/mL) and vasicine (10–70 μg/mL). TAAp contained vasicine (81.8%) and harmine (2.9%) as major alkaloids, TAR contained mainly harmine (90.9%) and harmol (2.85%). The TASe of *P. harmala* mainly contained harmine and harmaline that reached the very high amounts of 37.9% and 48.75%, respectively (Table 3).

Table 3
Content (%) of major alkaloids in total alkaloid extracts of different parts of *P. harmala*.

	Concentrations of the major alkaloids (% w/w; mean ± SD; n = 3)		
	TAAp[a]	TASe[b]	TAR
Total alkaloids	1.86	5	1.75
Harmine	2.92 ± 0.03	37.9 ± 0.1	90.9 ± 0.6
Harmaline	n.d.[c]	48.75 ± 0.01	n.d.
Harmane	n.d.	n.d.	n.d.
Harmol	n.d.	n.d.	2.85 ± 0.01
Harmalol	n.d.	n.d.	n.d.
Vasicine	81.8 ± 0.9	n.d.	n.d.

[a] TAAp: total alkaloid of aerial parts; TASe: total alkaloid of seeds; TAR: total alkaloid of roots.
[b] the alkaloids quantification of fruits alkaloid extract was similar to alkaloid extract of seeds.
[c] not determined.

4. Discussion

Chemotherapy plays an important role in the prevention and treatment of cancer; but its efficiency is restricted by cell resistance to the drugs, requiring constant improvement of treatments. New classes of chemotherapeutic agents have been continuously developed, among which naturally occurring compounds have increasingly attracted interest as lead compounds for drug development [5,27]. The first group of agents to advance into clinical use were the *Vinca* alkaloids, vinblastine and vincristine isolated from *Catharanthus roseus* [28].

P. harmala is widely reported, in traditional medicine, for the treatment of many diseases, including cancer [17]. Cytotoxic effects of the raw extract of seeds of this plant, such as aqueous, hydro-alcoholic and methanolic extracts, were previously reported, indicating a considerable cytotoxic potential [8,20,29–33].

The high alkaloid content of *P. harmala* extracts [7] prompted us to investigate their potential cytotoxicity; to our best knowledge, the activities of aerial parts, fruits and roots of *P. harmala* against several cancer cell lines *i.e.* A549, U373, Hs683, MCF7, B16F10 and SKMEL-28 have not been investigated so far. All total alkaloid extracts of fruits, seeds, roots and aerial parts decreased the viability of all analyzed cancer cell lines in a dose-dependent manner. Particularly, TAR induced a significant decrease on cancer cell viability. The results suggest that alkaloids are a major class of compounds in different parts of *P. harmala* and probably responsible for the observed cytotoxic effects. The differences between the activities of the different parts of the plant strongly depend on their alkaloids composition; also, a synergic effect of the different alkaloids cannot be excluded.

An interesting finding in the present study is that alkaloids extracted from our *P. harmala* samples inhibit the viability of the mammary cancer cell line (MCF7) at concentration (IC_{50}) lower than the IC_{50} value reported for the seeds total alkaloid extracts obtained from an Iranian *P. harmala* (MCF7 cell line; IC_{50}, 25 μg/mL) [18]. The raw alkaloid extracts of seeds have also been recently reported to inhibit the viability of MDA-MB-231 breast cancer cell (IC_{50}, 30 μg/mL) [34]; also, quinazoline alkaloids purified from seeds have been shown active on human gastric cancer cells (IC_{50} > 84 μmol/L) [20]. Consequently, the potent effect of TAAp extract could be related to its high content in vasicine.

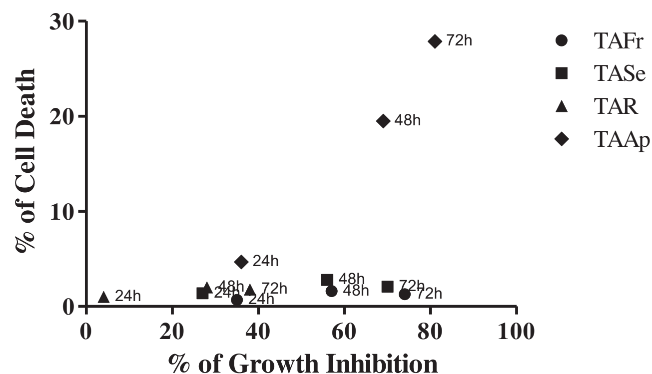

Fig. 4. Illustration of the % of cell death induced by each alkaloid extract under study (quantitative videomicroscopy analysis) in relation to the growth inhibitory effects induced by these extracts. Cell cultures have been monitored in triplicate for each experimental condition for 24 h, 48 h and 72 h. TAFr: total alkaloid of fruits; TASe: total alkaloid of seeds; TAR: total alkaloid of roots; TAAp: total alkaloid of aerial parts.

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Quantitative videomicroscopy indicated that the alkaloid extracts exhibit a cytostatic or cytotoxic effect on A549 lung cancer cell line, depending on the tested extract; the cytotoxic activity could be ascribed to the high concentration of the quinazoline alkaloid, vasicine, in the aerial parts extract.

In conclusion, total alkaloid extracts from aerial parts, fruits, seeds and roots of *P. harmala* exert potent cytotoxic and/or cytostatic effects toward several multi-drug resistant cancer cell lines. In a lung carcinoma A549 cell line, fruits, seeds and roots extract demonstrated mostly cytostatic effect whereas a cytotoxic activity was observed with aerial parts extract. The promising data of *P. harmala* alkaloid extracts may support the traditional claims regarding its anticancer uses; these could be helpful in providing new anticancer agents against chemo-resistant cancers as well as new leads for drug discovery; however the psychotropic activities of these alkaloids may limit their clinical use and pharmacomodulation may be necessary to dissociate the 2 types of activities. All the alkaloids extracted from different parts of the plant should be further investigated for *in vitro* cytotoxic activity, structure-activity relationships and *in vivo* anticancer studies.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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