Cytotoxicity of alkaloids isolated from *Peganum harmala* seeds

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Abstract: *Ethnopharmacological relevance: Peganum harmala* is used in traditional medicine to treat a number of diseases including cancer. Our preliminary studies show that the alkaloidal extract of PH seed is cytotoxic to several tumor cell lines *in vitro* and has antitumor effect in a tumor model *in vivo.*

The present investigation was aimed at extending our previous studies in identifying the components in *P. harmala* seed-extract responsible for the cytotoxic effects, and study the cytotoxic and antiproliferative activity of isolated alkaloids and total alkaloidal fraction (TAF) in several tumor cell lines.

Four alkaloids: harmalicidine, harmine, peganine (vasicine) and vasicinone were isolated from the *P. harmala* seed-extract and their activity and that of TAF were tested a) for their cytotoxic activity against four tumor cell lines [three developed by us by chemical-induction in Wistar rats: 1) Med-mek carcinoma ; 2) UCP-med carcinoma ; 3) UCP-med sarcoma] ; and 4) SP2/O-Ag14, and b) for antiproliferative effect on cells of Jurkat, E6-1 clone (inhibition of incorporation of {^{3}H-thymidine} in cellular DNA).

The alkaloids and TAF inhibited the growth of tumor cell lines to varying degrees; Sp2/O-Ag14 was the most sensitive, with IC_{50} values (concentration of the active substance that inhibited the growth of the tumor cells by 50%) ranging between 2.43 µg/mL and 19.20 µg/mL, while UCP-med carcinoma was the least sensitive (range of IC_{50} = 13.83 µg/mL to 59.97 µg/mL). Of the substances evaluated, harmine was the most active compound (IC_{50} for the 4 tumor cell lines varying between 2.43 µg/ml and 18.39 µg/mL), followed by TAF (range of IC_{50} = 7.32 µg/mL to 13.83 µg/mL); peganine was the least active (IC_{50} = 50 µg/mL to > 100 µg/ml).

In terms of antiproliferative effect, vasicinone and TAF were more potent than other substances: the concentration of vasicinone, and TAF needed to inhibit the incorporation of {^{3}H-TDR} in the DNA cells of Jurkat, E6-1 clone by 50% (IC_{50}) were 8.60 ± 0.023 µg/mL and 8.94 ± 0.017 µg/mL, respectively, while peganine was the least active (IC_{50} >100 µg/mL). The IC_{50} values for harmalacidine (27.10 ± 0.011 µg/mL) and harmine (46.57 ± 0.011 µg/mL) were intermediate. The harmala alkaloids inhibited the growth of four tumor cell lines, and proliferation of Jurkat cells with varying potencies. Harmine was the most potent in inhibiting cell growth, and vasicinone was most active as antiproliferating substance. The TAF had significant cytotoxic as well as antiproliferating activity.

Keywords: *Peganum harmala*; harmine; harmalacidine; peganine (vasicine); vasicinone; alkaloids; murine tumor cells; cytotoxicity; antiproliferating activity; anticancer activity; phytotherapy.

INTRODUCTION

Cancer in its various forms is a major health problem worldwide. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 (Ferlay *et al*., 2010; Jemal *et al*., 2011). The treatment of cancer includes surgery, radiation therapy and chemotherapy. Most of the available anticancer drugs are expensive, do not cure cancer and have serious adverse effects. Therefore, a majority of cancer patients (up to 80%) look towards alternate and complementary medicine as a primary or adjuvant therapy (Cassileth and Deng, 2004; Smithson *et al*., 2012). Phytotherapy is an alternate modality in the treatment of cancer (Miller *et al*., 2008; Dennis *et al*., 2009; Shu *et al*., 2010), since, in most cases plant-derived products are readily available, are relatively less expensive, less likely to cause dependency, and have low potential for serious side effects.

From ancient times, *Peganum harmala* (Syrian rue, harmal, esphand, etc.), a herbaceous perennial of the family Zygophyllaceae native to countries around the Mediterranean sea, central Sahara, the Middle East, India, Pakistan, south Australia, and western United States, has been used in traditional medicine for the treatment of variety of ailments, including cancer, depression, hallucinations, leishmaniasis, inflammation, malaria, and as an emmenagogue and abortifacient (Mahmoudian, *et al*., 2002 ; Chen *et al*., 2004 ; Bremner *et al*., 2009).

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In Morocco, *P. harmala* seed is used as a powder, decoction, macerate or infusion for treatment of fever, diarrhea, abortion, subcutaneous tumors, and a number of other ailments (Lamchouri *et al*., 1999; Farouk *et al*., 2008). *P. harmala* seed has been shown to have antimicrobial, antifungal, antiviral (Rashan *et al*., 1989; Shahverdi *et al*., 2008), central nervous system stimulant, analgesic, vasorelaxant (Berrougui *et al*., 2006b ; Farouk *et al*., 2008), and wound-healing (Derakhshanfar *et al*., 2010) properties. An extract of the seeds has been used to treat certain dermatoses (El-Saad El-Rifae, 1980) in humans, and theileiosis in animals (Mirzaei, 2007). In addition, the seed extract has been shown to have hypoglycemic activity in streptozotocin induced-diabetic rats (Singh *et al*., 2008). The pharmacological and the reported therapeutic effects of *P. harmala* may be attributed to a number of phytochemicals that have been isolated from *P. harmala* seeds, such as the β-carboline alkaloids (harmine, harmaline, harmalacidine, harmol, harmalol, and tetrahydroharmine; Astulla *et al*., 2008; Lamchouri *et al*., 2008; Pulpati *et al*., 2008; Herraiz, 2010), and the quinazoline alkaloids [vasicine (peganine), vasicinone and deoxyvasicinone; Astulla *et al*., 2008; Lamchouri *et al*., 2008; Pulpati *et al*., 2008)]. Pharmacological studies show diverse activities of the constituents of *P. harmala* seeds, including: a) inhibition of acetylcholine esterase (Wang *et al*., 2009); b), inhibition of monoamine oxidase (Herraiz, *et al*., 2010); c) antidepressant (Mansouri and Farzin, 2009); d) antiplatelet (Im *et al*., 2009), e) vasorelaxant (Berruoughi *et al*., 2006b; Astulla *et al*., 2008); f) anti-oxidant (Berrougui *et al*., 2006a), g) hypotensive (Dewick, 2009), and h) bronchodilatory (Dewick, 2009) activities. Some of the β-carboline alkaloids also have antimicrobial (Shahverdi *et al*., 2008), anti-AIDS (Ishida *et al*., 2001), antifungal (El-Saad El-Rifaie, 1980), antipruritic (El-Saad El-Rifaie, 1980), antiviral (Hudson, *et al*., 1986), and antiplasmodial (Astulla *et al*., 2008) activity.

P. harmala has also been used in traditional medicine in cancer therapy. For example, an extract of *P. harmala* is one of the main components of an ethnobotanical preparation used in the treatment of cancer in Iran (Sobhani *et al*., 2002). Powdered seeds of *P. harmala* are used in herbal formulas of Chinese medicine to cure digestive tract tumors (Chen *et al*., 2004; Song *et al*., 2006). In Morocco, the seed powder is sometimes used on skin and subcutaneous tumors (Lamchouri *et al*., 2000).

The antitumor and cytotoxic effects of the *P. harmala* alkaloids in various malignancies have also been described (Ishida *et al*., 1999). Harmine and harmaline have been shown to be cytotoxic (Zaker *et al*., 2007), while harmine, harmaline and harmalol have antiproliferative activity (Ayoub *et al*., 1994; Zaker *et al*., 2007).

Our preliminary investigations indicated that the total alkaloidal fraction (TAF) of *P. harmala* seeds has *in vitro* cytotoxic activity against several cancer cell lines (Lamchouri *et al*., 1999; Lamchouri *et al*., 2000). In addition, an *in vivo* study in syngenic BALB/c mice grafted with the subcutaneous neoplasmic cell line Sp2/O-Ag14 showed that the aqueous and alcoholic extracts of *P. harmala* seeds have potent antitumor and antiproliferative activity (Lamchouri *et al*., 1999). These results prompted us to isolate and identify the active principle(s) in the methanolic extract of *P. harmala* seeds which may be responsible for the cytotoxic and antitumor activities. In this regard, four alkaloids, I (harmalacidine), II (harmine), III [peganine (vasicine)], and IV (vasicinone), were isolated (by chromatography on silica gel), and their identities were confirmed (Lamchouri *et al*., 2008).

In the present study, we investigated whether the above four alkaloids isolated from the seed extract of *P. harmala* were responsible for the observed antitumor and cytotoxic activity of TAF. In this respect, the cytotoxic and antiproliferative activities of the pure alkaloids (I-IV) and TAF were determined in several cancer cell lines.

MATERIALS AND METHODS

Plant materiel

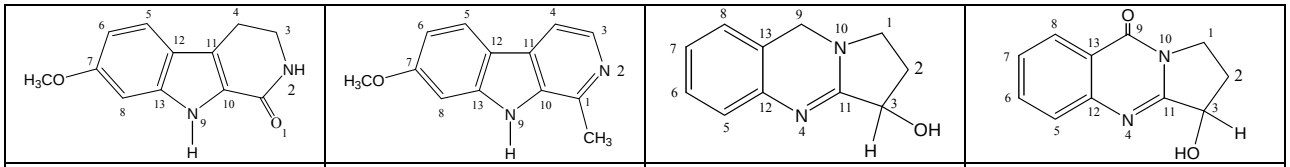
The whole plant with the seeds of *P. harmala* was collected from the Figuig region of eastern Morocco, in July-August, and identified and authenticated by Professor M. Fennane of the Scientific National Institute (Rabat). The seeds of *P. Harmala* were further authenticated by Dr A. Bennabidine, botanist (École Nationale Forestière d'Ingénieurs de Rabat-Salé). A voucher specimen of the seeds (AT-09) was deposited in the herbarium of the Polydisciplinary Faculty of Taza.

Tested materials

The alkaloidal fraction was isolated (overall yield = 1.2%) from the methanolic extract of *P. harmala* seeds as described previously (Lamchouri *et al*., 1999; Lamchouri *et al*., 2000). Four compounds (I-IV, fig. 1) were isolated from the alkaloid fraction (by chromatography) with yields of 52%, 10%, 4% and 1.3%, respectively, and identified by spectroscopic techniques (nuclear magnetic resonance and mass spectrometry) (Lamchouri *et al*., 2008), as I) harmalacidine, II) harmine, III) peganine (vasicine), and IV) vasicinone (4). Compound I and II are β-carboline alkaloids, while compounds III and IV are quinazoline alkaloids.

Tumor cell lines

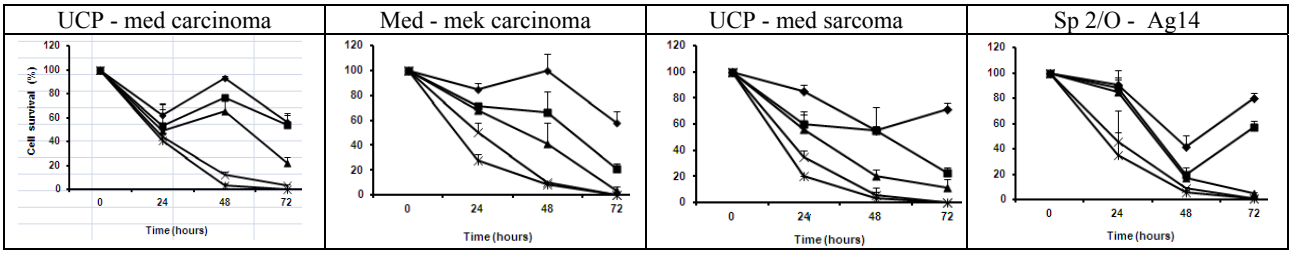
Cytotoxicity of the four compounds (I-IV) isolated from the alkaloid fraction as well as TAF was evaluated in four cell-lines, three of them obtained from chemically-induced cancer in Wistar rats: (a) Med-meek carcinoma, obtained



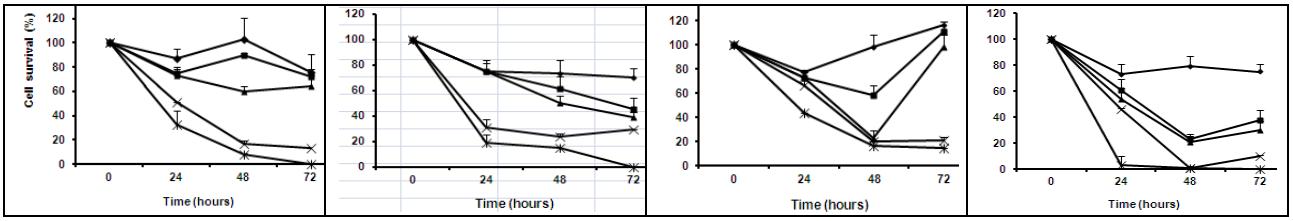
Harmalacidine (I)	Harmine (II)	Peganine (Vasicine ; III)	Vasicinone (IV)

Fig. 1: Structures of compounds **I-IV** isolated from the seeds of *Peganum harmala* [harmalacidine (I), harmine (II), peganine (vasicine, III), and vasicinone (IV).

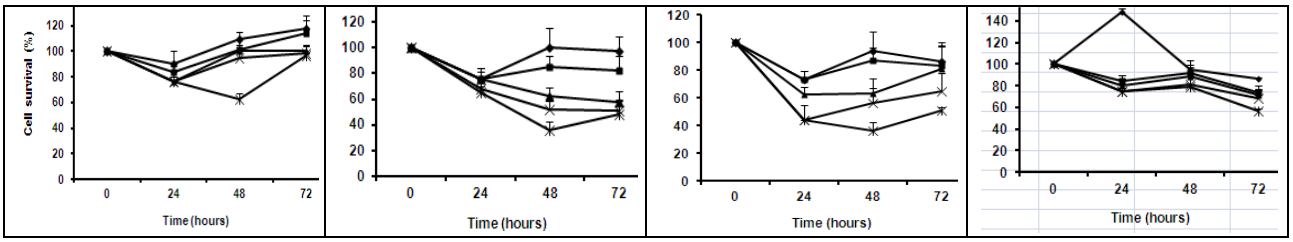
Harmine



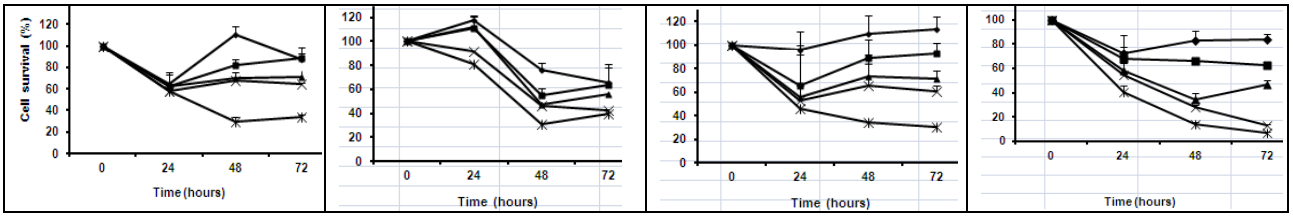
Harmalacidine



Peganine (vasicine)



Vasicinone



Total alkaloidal fraction

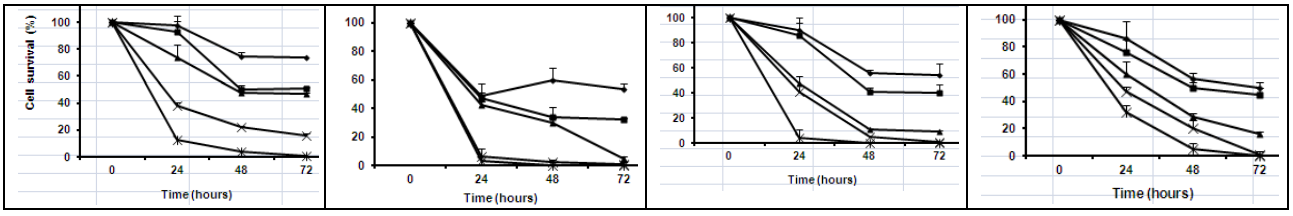


Fig. 2: Effect of *Peganum harmala* alkaloids on the survival of tumor cells.
Legend: concentration of the alkaloids; All values, expressed as mean ± SEM of five set of experiments, are significantly different when compared with controls ($p<0.05$); details of the experimental procedure are given in the text.

from a hepatocarcinoma on cirrhosis induced by carbon tetrachloride; (b) UCP-med carcinoma obtained from a hepatocarcinoma induced by diethylnitrosamine; (c) UCP-med sarcoma obtained from a fibrosarcoma induced by 3-methylcholanthrene; and the fourth one (d) murine cell line, SP2/O-Ag14, a non-IgG-secreting or synthesizing line derived from the fusion of BALB/c mouse spleen and the mouse myeloma P3X63Ag (Schulman *et al*., 1978). The cell lines, 'a-c', were established in our laboratory (Lamchouri *et al*., 2001). The Jurkat, E6-1 clone was established by Dr. A. Weiss (Howard Hughes Medical Institute, University of California, San Francisco) by cloning the Jurkat-FHCRC line, and was obtained from Service de Pharmacologie de l'hôspital Intercommunal de crétéil, France.

Cytotoxicity assays

In vitro cytotoxicity assay was carried out as described previously (Lamchouri *et al*., 2000) using trypan blue (dye exclusion) method (see below).

Tumor cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 2% L-glutamine and 5 mg/L of each of the antibiotics, gentamicin, penicillin, and streptomycin to prevent bacterial and mycoplasmal contamination. Cells were maintained in a 5% CO_2 - 95% O_2 incubator at 37°C, and grown to confluence for 2 to 3 days and adjusted to a concentration of 10^5 cells/mL with fresh medium. Initial cultures of 10 mL were prepared in 25 Ml flasks before application of treatments.

The alkaloids were solubilized in 0.2 % dimethylsulfoxide (DMSO) and applied to the cell cultures at final concentrations of 5, 10, 25, 50 and 100 µg/mL. The alkaloid solutions were added to each culture after adjustment to the same volume of DMSO. Control experiments were carried out with the same amount of DMSO without the alkaloids. An additional normal control was carried out with an equivalent volume of culture medium instead of DMSO.

All treatments were carried out in five replicates. The cultures were maintained under normal incubation conditions and were examined daily under an inverted microscope. Cell counts were made on 0.1 mL samples taken from the cultures after vigorous shaking to monitor cell viability using the trypan blue colorimetric method (Phillips, 1973). In brief, cells were trypsinized and stained with trypan blue (prepared by mixing 4% trypan blue diluted 1:1 with phosphate buffered saline). The number of cells which excluded trypan blue (considered viable) was counted in a Burker chamber within 5 min of staining. The concentration of each substance needed to produce 50% inhibition (IC_{50}) was determined from the dose-effect curves after 48 hrs of incubation.

Antiproliferative assay

The antiproliferative effect of the alkaloids was evaluated by determination of inhibition of tritiated-thymidine {[^{3}H]-TDR} incorporation into the DNA of cells of Jurkat, E6-1 clone. The cells (1×10^6 to 3×10^6) were cultured in RPMI (Roswell Park Memorial Institute) - 1640 medium, with 10% fetal calf serum, L-glutamine (0.1%) and antibiotics (gentamicin 0.1% - penicillin/ streptomycin 0.1%), in plates with round bottom cavities. Then 200 µL of the test substances at concentrations of 5, 10, 25, 50 and 100 µg/mL and (^{3}H-methyl-thymidine, 4 pCi/mL, specific activity 720 mCi/mmole) were added, and the plates were incubated for 48 hr at 37°C with 5% CO_2 – 95% O_2 and 5% relative humidity.

After incubation, cells were harvested and deposited on a glass fiber filters using Shatron multiple cell harvester. The filters were dried for 24 h at room temperature and transferred to miniscintillation vials. After adding 10 mL of scintillation fluid to each vial, radioactivity was determined by using a liquid scintillation spectrometer (Tri-Carb Scintillation Counter). Three separate sets of control Jurkat E6-1 clone containing the compounds or the solvent were used in each assay. Data were obtained as counts/minute and the mean of each triplicate was calculated.

For each substance tested, the concentration inhibiting the incorporation of [^{3}H]-TDR in Jurkat cells by 50% (IC_{50}) was determined (after 48 hours of treatment), from the dose-response curves.

STATISTICAL ANALYSIS

Statistical analysis of the data was conducted using Student's t-test and the values are expressed as mean ± SEM for n = 5 (cytotoxicity assays) and n = 3 (antiproliferative assays). A p value of less than 5 per cent was considered statistically significant. The plots of dose-effect were analyzed to determine 50% inhibition (IC_{50}) using the program MICROPHARM (Urien, 1995).

RESULTS

Cytotoxicity of P. harmala alkaloids to tumor cell lines

DMSO used as a solvent did not have a significant effect on cellular growth when administered alone at concentrations used in our experiments as compared with the normal control.

The results of the cytotoxicity study of TAF, the two β-carboline alkaloids [harmalacidine (I) and harmine (II)], and the 2 quinazoline alkaloids [peganine (III; vasicine) and vasicinone (IV)], isolated from the seed extract of *P. harmala* on the 4 murine tumor cell lines, are expressed as IC_{50} values, and are based upon cell survival data at various concentrations of the alkaloids (fig. 2); these are summarized in table 1.

Table 1: Cytotoxicity of total alkaloidal fraction and compounds I-IV isolated from *Peganum harmala* seeds in 4 tumor cell-lines

Tumor cell line	Total alkaloidal fraction	I	II	III	IV
		IC_{50} (µg/mL)*			
Med-mek carcinoma	7.74 ± 0.017	17.72 ± 0.022	14.40 ± 0.013	52.24 ± 0.017	25.32 ± 0.035
UCP-med carcinoma	13.83 ± 0.017	28.93 ± 0.008	18.39 ± 0.013	> 100	59.97 ± 0.022
UCP-med sarcoma	7.32 ± 0.017	17.60 ± 0.013	6.48 ± 0.022	52.36 ± 0.022	64.79 ± 0.017
Sp2/O-Ag14	9.24 ± 0.026	7.96 ± 0.017	2.43 ± 0.062	> 100	19.20 ± 0.017

Data shown are mean ± SEM of five independent experiments.
I = harmalacidine ; II = harmine; III = peganine (vasicine) ; IV = vasicinone
*Cytotoxicity, expressed as IC_{50} for each cell line, is the concentration of the compound that inhibits cell multiplication by 50% after 48 hours of treatment [calculated from dose-response curves (fig. 2)].

Table 2: Effect of total alkaloidal fraction and compounds I-IV isolated from *Peganum harmala* seeds on proliferation of cells of Jurkat, E6-1 clone

Cell line	Alkaloidal fraction	I	II	III	IV
		IC_{50} (µg/mL)*			
Jurkat, E6-1 clone	8.94 ± 0.017	27.10 ± 0.011	46.57 ± 0.011	> 100	8.60 ± 0.023

Data shown are mean ± SEM of three independent experiments.
I = harmalacidine; II = harmine; III = peganine (vasicine); IV = vasicinone
*Inhibition of proliferation, expressed as IC_{50}, the concentration of a substance that inhibits the incorporation of tritiated thymidine in the cells of Jurkat, E6-1 clone, by 50% after 48 hours of treatment

In terms of cytotoxicity, the Sp2/O-Ag14 was found to be most sensitive of the 4 tumor cell lines (range of IC_{50} = 2.43 µg/mL to19.20 µg/mL for the substances tested), while UCP-med carcinoma was the least sensitive (range of IC_{50} = 13.83 µg/mL to 59.97 µg/mL). TAF (range of IC50 = 7.32 µg/mL to 13.83 µg/mL) and harmine (range of IC_{50} for the 4 tumor cell lines = 2.43µg/ml to 18.39 µg/mL) were the most active substances, especially for the Sp2/O-Ag14 cell line (IC_{50} of 2.43 ± 0.062 µg/mL). On the other hand, peganine (vasicine) was the least active, having minimum effect on the growth of Sp2/O-Ag14 and carcinoma cells (IC_{50} >50 µg/ml to > 100 µg/ml).

Antiproliferative activity of P. harmala alkaloids

The effect of TAF and the 4 pure alkaloids (I-IV) on the incorporation of {^{3}H-TDR} in the DNA of the cells from the Jurkat, E6-1 clone is summarized in table 2 (presented as IC_{50}). The inhibition of {^{3}H-TDR} incorporation by vasicinone, harmalacidine, and harmine was dose-dependent; the calculated IC_{50} were 8.60 ± 0.023 µg/mL for vasicinone, 8.94 ± 0.017 µg/mL for TAF, 27.10 ± 0.011 µg/mL for harmalacidine and 46.57 ± 0.011 µg/mL for harmine; TAF was as potent as vasicinone, while peganine was the least active alkaloid.

DISCUSSION

The results of the present investigation showing cytotoxicity of the four alkaloids and TAF in 4 tumor cell lines are in agreement with previous studies which show that some of the alkaloids isolated from *P. harmala* seed are cytotoxic to a number of tumor cell lines. For example, harmine exhibited cytotoxity to a) HL60 leukemic cells (Jahaniani *et al*., 2005; Zaker *et al*., 2007); b) K562 cells (Jahaniani *et al*., 2005); c) KB, A549, CAKI-1, 1A9 and HEL cells (associated with cancers of nasopharynx, lung, kidney, breast and ovaries, respectively) as well as to three drug resistant cell lines KB-7d, KB-VIN and KB-CPT (Ishida *et al*., 1999); d) breast cancer cells (Ma and Wink, 2010). Harmine and harmaline inhibited the growth of three human cancer cell lines, UACC-62 (melanoma), TK-10 (renal) and MCF-7 (breast) (Berrougui *et al*., 2005). Harmine and its derivatives exhibited significant cytotoxicity in several tumor cells lines [non-small cell lung carcinoma (PLA-801), liver carcinoma (HepG2 and Bel-7402), gastric carcinoma (BGC-823), cervical carcinoma (Hela), and colon carcinoma (Lovo) (Cao *et al*., 2005a)].

A patent application (U.S. Patent No. 20080069899; accessed June 2012) describes the results of *in vitro* studies of cytotoxicity of harmine, harmalacidine and peganine (vasicine) in KB (epithelioma of nasopharnx), K562 (human leukemia), Jurkat (leukemia), U937 (myeloma), HT29 (colon cancer) and HBMEC (immortalized human bone marrow endothelial cells), and *in vivo* studies in severe combined immunodeficient mice xenografted with human tumor cells (breast, colon, prostate, ovarian, pancreatic, hepatic, etc.) treated orally or intravenously with or without standard anticancer drugs.

Our results are in agreement with previous *in vitro* studies showing that harmine was more potent than harmalacidine, with peganine being least cytotoxic

(www.freepatentsonline.com/y2008/0069899.html; accessed August 2010). The higher activity of harmine (compared to other compounds studied) may be explained on the basis of its lipophilic nature and its avid binding to DNA (Nafisi *et al*., 2010a) and RNA (Nafisi *et al*., 2010b), inhibition of DNA topoisomerases and blockade of DNA synthesis in the S phase of the cell cycle (Sobhani *et al*., 2002; Song *et al*., 2004), causing apoptosis and necrosis in human SGC-7901 (Song *et al*., 2006) and HepG2 cells (Chen *et al*., 2004; Song *et al*., 2006; Cao *et al*., 2011), and inhibition of certain cyclin-dependent kinases, which regulate cell proliferation cycle (Song *et al*., 2004). Further, the higher activity of TAF compared to the purified alkaloids may be due the presence of other alkaloids in *P. harmala* seeds (such as harmol, harmaline, norharman, etc.) not tested in this study, and/or some non-alkaloidal substance(s) in the crude fraction, such as flavonoid glycosides (Sharaf *et al*., 1997) and anthraquinones (Pitre and Srivastava, 1987; Wang *et al*., 2008), which are known to be present in *P. harmala*. A synergism or potentiation of the anti-tumor activity by different components of TAF may also explain the higher activity, as has been demonstrated for several other anticancer compounds (Gu *et al*., 2000; Papadopoulou *et al*., 2003 Hernlund *et al*., 2008).

In terms of antiproliferative activity, vasicinone and TAF were more potent than other alkaloids, while peganine was the least active. Previously, harmal alkaloids have been shown to inhibit proliferation of a number of human tumor cells: HeLa (cervical), MCF-7 (breast), SW480 (colon), and HL60 leukemic cells (Song *et al*., 2004; Zaker *et al*., 2007). The mechanism of antiproliferating action of the harmala alkaloids may be similar to those described for cytotoxicity. The higher activity of TAF may be due to factors described above.

Although, the *in vitro* results may not be translated to antineoplastic effect *in vivo* [because of several factors, including transport, binding and metabolism of the active components, not occurring in cell culture), and the possible differences in the nature of the tumor cells *in vitro* and *in vivo*], the *in vitro* testing is the first step in determining if certain substances have the potential to be developed as anticancer agents. A substance with no cytotoxic effect *in vitro* is less likely to be active *in vivo*, unless it is metabolized to an active compound. Results from the present study suggest that harmine and harmalacidine should be tested against several other types of human tumors *in vitro* and when explanted *in vivo* in experimental animals in attempts to develop anticancer compound(s).

In conclusion, the present study of the cytotoxic and antiproliferative effects in experimental models of cancer show that the TAF and three of the four alkaloids (harmine, harmalacidine and vasicinone) isolated from the seed extract of *P. harmala* have significant *in vitro* cytotoxic activity in tumor cell lines, and antiproliferative activity on Jurkat line, E6-1 clone. The results of the present investigation and previous studies suggest that *P. harmala* seed alkaloids have significant antitumor activity, and the β-carboline structure could be an important basis for the design and synthesis of new antitumor drugs. Further studies are needed to isolate and identify additional potent cytotoxic substances in the TAF, to determine the mechanism(s) of action of the alkaloids, and to find out if the *in vitro* studies of cytotoxicity and inhibition of cell proliferation can be duplicated *in vivo* in inhibiting human tumors xenografted in mice.

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