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Cytotoxic activity of methanolic extract and two alkaloids extracted from seeds of *Peganum harmala* L.

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Abstract

Objective: To study the cytotoxic activity of *P. harmala*. Materials and method: The alkaloids harmine and harmaline have been isolated from a methanolic extract from the seeds of *P. harmala* L. and have been characterized by spectroscopic-Mass and NMR methods. The cytotoxicity of the methanolic extract and both alkaloids has been investigated in the three human cancer cell lines UACC-62 (melanoma), TK-10 (renal) and MCF-7 (breast) and then compared to the positive control effect of the etoposide. Results and conclusion: The methanolic extract and both alkaloids have inhibited the growth of these three cancer cell-lines and we have discussed possible mechanisms involved in their cytotoxicity.

Keywords: *Peganum harmala,* harmine, harmaline, cytotoxicity, TK-10, MCF-7, UACC-62.

1. Introduction

Peganum harmala L. (Zygophyllaceae), the so-called harmal, grows spontaneously in uncultivated and steppes areas in semiarid and pre-deserted regions in south Spain and South-East Morocco [1]. Harmala alkaloids are distributed widely in some medicinal plants and are found endogenously in mammalian tissues. Harmala alkaloids have a wide spectrum of pharmacological actions in the central nervous system such as tremorogenesis [2], hypothermia [3,4], hallucinogenesis [5,6], central monoamine oxidase inhibition [7-9], convulsive or anticonvulsive actions and binding to various receptors including 5-HT receptors and the benzodiazepine binding site of $GABA_A$ receptors [10]. In addition, these compounds also have antioxidative [11], platelet aggregation inhibitory and immunomodulatory effects [12-13]. There are also some reports concerning the cardiovascular actions of harmala alkaloids, such as systemic arterial blood pressure and total peripheral vascular resistance reduction by harmine, or an *in vivo* vasorelaxant effect

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of Harman [14]. In addition, we reported in a recent work the vascular relaxant effect of a methanolic extract from seeds of *P. harmala* L. (MEP) [15].

In the present study we isolated and characterize spectroscopycally two alkaloids from such extract and evaluated their cytotoxic activity in three human cancer cell lines.

2. Material and methods

2.1 Plant

Peganum harmala L. (Zygophyllaceae) fresh seeds were collected from the medium of atlas region (Morocco), in May 2002 and botanically identified by botanical section of (U.F.R: Naturals Products), Faculty of Medicine and Pharmacy. Rabat; where a voucher specimen is preserved.

2.2 Extraction of methanolic extract (ME) and natural compound

Fresh and powdered seeds were successively extracted in a soxhlet apparatus with Petrol-ether (60-80°C), $CHCl_3$, EtOAc and MeOH. The obtained extract was concentrated under reduced pressure to yield dry residues: 0.67, 1.42, 2.8, 31% (w/w), respectively. The naturals compound (Harmaline and Harmine) were extracted from the last fraction (Methanolic extract); this fraction was chromatographed on silica gel column, and the elution was started with $CHCl_3$ following with $CHCl_3$ containing increasing amount of MeOH.

All fractions obtained from this silica gel column were subjected to TLC (thin layer chromatography) examination using $CHCl_3$-MeOH (9:1). Fractions with similar migration times were combined. Harmine and harmaline were isolated by crystallisation from their fractions using in $CHCl_3$ and MeOH respectively. the compounds were analysed by TLC, ^{1}H-NMR, ^{13}C-NMR (Nuclear Magnetic Resonance) and mass spectra.

2.3 Human tumour cell line

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. They were kindly provided by Dr. G. Cragg, Department of NCI, Maryland, USA.

The human tumour cytotoxicities were determined following protocols established by the National Cancer Institute, National Institute of Health [16]. TK-10, MCF-7 and UACC-62 cell lines were cultured in RPMI 1640 medium (Biowhittaker) containing 20% fetal calf serum, 20mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity.

2.4 Cytotoxicity assay

For the assay, cells were detached with 0.1% trypsin-EDTA (Sigma) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final concentrations of 15×10^4 5×10^4 and 100×10^4 cells/ml for TK-10, MCF-7 and UACC-62 respectively. 100µl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment.

After 24 h the cells were treated with the serial concentrations of compounds or extracts. They were initially dissolved in an amount of 100% DMSO (10 mM) and further diluted in medium to produce 5 concentrations. 100µL/well of each concentration was added to the plates to obtain final concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M for the compounds and 250, 25, 2.5, 0.25 and 0.025µg/mL for the extract. The DMSO concentration for the tested dilutions was not greater than 0.25% (V/V), the same as in solvent control wells. The final volume in each well was 200µl. The plates were incubated for 48 h.

Table I.
^{1}H (A)- and ^{13}C (B)-NMR data of harmaline and harmine in CDCl$_3$ and DMSO, respectively.

A.	Harmaline (200 MHz, CDCl$_3$)				Harmine (200 MHz, DMSO)		
H	**δ [ppm]**	**multiplicity**	**J [Hz]**	**H**	**δ [ppm]**	**multiplicity**	**J [Hz]**
NH	11.54	s		NH	11.42	s	
H-8	7.04	d	1,9	H-8	7.00	d	2.1
H-6	6.80	dd	1,9 /8,9	H-6	6.83	dd	2.1/8.6
H-5	7.42	d	8,9	H-5	8.04	d	8.6
H-4	3.14	m		H-4	7.79	d	5.3
H-3	3.88	m		H-3	8.14	d	5.3
OCH$_3$	3.83	s		OCH$_3$	3.86	s	
CH$_3$	2.99	s		CH$_3$	2.71	s	

B.

C	Harmaline (50 MHz, CDCl$_3$)	Harmine (50 MHz, DMSO)
1	161.889	14.934
2	-	-
3	41.839	137.762
4	19.908	111.947
5	122.212	122.625
6	115.754	109.068
7	164.575	160.074
8	94.049	94.57
9	-	-
10	125.029	134.534
11	119.038	114.833
12	125.549	127.206
13	144.201	141.274
CH$_3$-1	19.05	20.344
CH$_3$O-7	55.704	55.319

(d = doublet, dd = doublet doublet, m = multiplet,
s = singulet)

Sulphorhodamine B method: This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. After incubating 48 h, adherent cell cultures were fixed in situ by adding 50μl of cold 50% (W/V) trichloroacetic acid (TCA) and incubating for 60 min. at 4°C. The supernatant is then discarded, and the plates are washed five times with deionised water and dried.

One hundred ml of SRB solution (0.4% wt/vol in 1% acetic acid) is added to each microtiter well and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. Then the plates were air-dried. Bound stain is solubilized with Tris buffer, and the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 492 nm.

At the end, IC$_{50}$ values were calculated and at least three independent experiments were carried out for each compound or extract. Data are given as the mean ± SEM.

3. Results and discussion

3.1. Chemistry

Harmine and harmaline were isolated by crystallisation after silica gel column separation. The TLC analysis revealed two spots (blue and violet) under UV light with Rf values 0.62 (harmine) and 0.36 (harmaline). This data are in agreement with those reported in the literature (0.64 and 0.35 respectively)[17]. Furthermore, the ^{1}H-NMR and ^{13}C-NMR data illustrated in Table 1 revealed that these compounds were harmine and harmaline (Fig 1).

Table 2
Cytotoxic activity (expressed as $IC_{50} \pm SEM$ values) of harmine, harmaline, methanolic extract where they were isolated, and the positive control (etoposide) on the human cancer cell lines TK-10, MCF-7 and UACC-62.

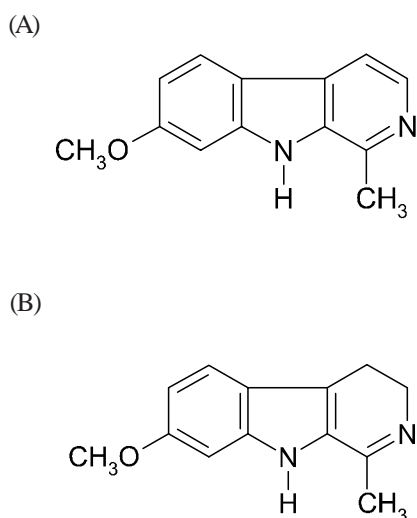|	n		TK-10	MCF-7	UACC-62
ME	3	IC_{50} (µg/mL)	38.3 ± 2.3	25.3 ± 4.2	26.6 ± 3.1
Harmine	3	IC_{50} (µM)	40.5 ± 6.8	29.3 ±3.5	18.0 ± 0.7
Harmaline	3	IC_{50} (µM)	27.5 ± 3.2	16.4 ± 2.5	11.1 ± 1.5
Etoposide	3	IC_{50} (µM)	9.95 ± 0.08	0.87 ± 0.21	1.13 ± 0.21

n: number of independent experiments.

(A)

(B)

Fig 1. Chemical structure of harmine (A) and harmaline (B).

3.2. Cytotoxicity assay

Although the role of nitric oxide in tumor biology remains incompletely understood, it is known to have both tumour promoting and inhibitory effects, presumed to be dependent on its local concentration within the tumor. The p53 tumor suppressor gene may influence its production, and NO appears to be pivotal in the angiogenic processs being necessary for solid malignant tumor development.

In addition, nitric oxide has been shown to induce cytostasis and cell cycle arrest on the human breast cancer cell line MDA-MB-231 [18,19]. Bearing in mind that the alkaloids harmine and harmaline have been shown to induce NO production from cultured rat aortic endothelial cells [14], we considered the possible anticancer effect of such alkaloids. Therefore, we assayed the cytotoxic activity, on the human cancer cell lines TK-10, MCF-7 and UACC-62, of these alkaloids as well as the plant extract where they were isolated, being the results depicted in Table 2.

The antineoplastic agent etoposide was taken as positive control for comparison with the tested compounds. The extract and the alkaloids were active on the three cancer cell lines at the recommended USA National Cancer Institute (NCI), although they were less active than etoposide. Harmaline's activity was slightly higher than the observed for harmaline, suggesting that the change of β-carboline to dihydro-β-carboline might be increasing the anticancer activity.

The alkaloids concentrations producing an inhibition of the growth by 50% (IC_{50}) on the three human cancer cell lines were similar to the concentrations required for the induction of NO production by such compounds [14]. Therefore, we might think that their anticancer action is mediated by NO production.

However, despite the fact that harmine showed less NO production activity that harmaline, its anticancer activity in our cancer cell lines was higher, suggesting the involvement of other mechanisms in its anticancer action.

In this way, Ayoub *et al.,* [20] have reported that harmalol (an indole alkaloid from P. *harmala*) inhibited the proliferation of K562-Leukaemic tumor cell line by inhibiting DNA synthesis and cell division. A previous work has shown that β-carbolines could intercalate into DNA, this effect may cause inhibition of DNA topoisomerases leading to a cytotoxic effect. Accordingly, Sobhani *et al.,* [21] have reported that *P. harmala* seeds extract inhibit human DNA topoisomerase I.

References

1. Hmamouchi M. (1999) Les plantes medicinales et aromatiques Marocaines, Enigma ed, 9954-8007-0-0: 450.

2. Lutes J, Lorden JF, Beales M, Oltmans GA. (1988) *Neuropharmacol.* 27: 849-855.

3. Bruinvels J, Sourkes TL. (1968) *Eur. J. Pharmacol.* 4: 31-39.

4. Abdelfattah AFM, Matsumoto K, Gammaz HAK, Watanabe H. (1995) *Pharmacol. Biochem. Behav.* 52 (2) : 421-426.

5. O'Hearn E, Molliver ME. (1993) *Neuroscience* 55 (2): 303-310.

6. Grella B, Dukat M, Young R, Teiler M, Herrick-Davis, Gauthier CB, Glennon RA. (1998) *Drug Alcohol depend.* 50: 99-107.

7. Nelson DL, Herbet A, Petillot Y, Pichat L, Glowinski J, Hamon M. (1979) *J. Neurochem.* 32: 1817-1827.

8. Fuller RW, Wong CJ, Hemrick-Luecke SK. (1986) *Life Sci.* 38: 409-412.

9. May T, Rommelspacher H, Pawlik M. (1991) J. Neurochem. 56: 490-499.

10. Rommelspacher H, Bruning G, Susilo R, Nick M, Hill R. (1985) *Eur. J. Pharmacol.* 109:363-371.

11. Tse SYH, Mak IT, Dickens BF. (1991) *Biochem Pharmacol.* 42: 4549-464.

12. Saeed SA, Farnaz S, Simjee RU, Malik A. (1993) *Biochem. Soc. Trans.* 21: 462S.

13. Li WK. (1996) J *Lanzhou Med Coll.* 22: 16-18.

14. Shi CC, Liao JF, Chen-Fu C. (2001) *Jpn. J. Pharmacol.* 85: 299-305.

15. Berrougui H, Herrera MD, Marhuenda E, Ettaib A, Hmamouchi M. (2002) *Therapie.* 57: 236-241.

16. Monks A, Scudeiro D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, CrOnise P, Vaigro-wolff A *et al.* (1991) *J. The National Cancer Institute* 83 (11): 757-766.

17. Christy M, Zaidi I, Nasir MA. (1995) *Fitoterapia* LXVI (1): 73-76.

18. Pervin S, Singh R, Chaudhuri G. (2001) *Proc. Natl. Acad. Sci.* USA. 98(6): 3583-8.

19. Brennan PA, Moncada S. (2002) *Ann. R. Surg. Engl.* 84(2): 75-78.

20. Ayoub MT and Rashan LJ. (1991) *Phyto-chemistry* 30: 1046-1047.

21. Sobhani AM, Ebrahimi SA, Mahmoudian M. (2002) *J. Pharm. Pharmaceut. Sci.* 5(1): 19-23.