Antitumor and Immunomodulatory Activities of *Peganum harmala* Extracts

فعاليات مستخلصات نبات الحرمل المضادة للاورام وكمعدلات مناعية

Moyassar B. H. Al-shaibani **Hiba T. H. Al-mafrachi**
College of Science/ Al-Nahrain University

ميسر باسل هادي الشيباني **هبة ثامر حسن المفرجي**
جامعة النهرين/ كلية العلوم

Abstract:

This study was designed to search the antitumor potential of *Peganum harmala* methanolic extracts and to evaluate their role in retardation the immunological side effects pre-and post-treatment which were carried out on 30 Albino male mice and included total and absolute count of leucocytes, micronucleus formation and phagocytic index of peritoneal cells. Our study showed that *P. harmala* derivatives have considerable antitumor activity with high efficacy in immobilization of the immunological side effects evoked following treatment with antitumor drug. In conclusion, *P. harmala* possess antitumor activity increases with concentrations increasing. Pre-treatment with these extracts exert higher retardation to the side effects than post-treatment.

المستخلص

صممت هذه الدراسة لبحث فعالية مستخلصات نبات الحرمل ضد الاورام وتقدير دورها في تقييد ظهور التأثيرات الجانبية المناعية الناتجة قبل و بعد العلاج بالأدوية المضادة للأورام والتي أجريت على 30 حيوانا من ذكور الفأر الأبيض والتي تضمنت العدد الكلي والتصنيفي لكريات الدم البيضاء ، تشكل النويات المايكروية ومعامل البلعمة . أثبتت الدراسة بأن المستخلصات الكحولية لنبات الحرمل لها القابلية لتثبيط نمو الخلايا السرطانية . من ناحية اخرى كان للمستخلصات الكحولية لنبات الحرمل تأثيرا ايجابيا واضحا في تقييد التاثيرات الجانبيه الناتجة عن العالجة بالادوية المضادة للاورام . نستنتج بأن نبات الحرمل يمتلك فعالية مضادة للاورام تتناسب طرديا مع التركيز. بالاضافة الى أن المعالجة السابقة بمستخلصات هذا النبات لها القابلية على كبح التأثيرات الجانبية الناتجة عن علاج الاورام أكثر من المعالجة اللاحقة .

Introduction

Peganum is a small genus belonging to the family Zygophylaceae and mainly distributed in the Mediterranean region. *Pegenum harmala* is the only species found growing wild in the middle and northern parts of Iraq. The plant is rich in alkaloids and contains up to 4% total alkaloids [1]. The principle alkaloids present are harmaline, harmine, harmalol and peganine [2].

There is an increasing interest in the discovery of novel antitumor agents from natural resources. The β-carboline alkaloids present in medicinal plants such as *Peganum harmala* have recently drawn attention due to their antitumor properties [3].

The pharmacologically active compounds of *P. harmala* are several alkaloids, which are found especially in the seeds and the roots. These include β-carbolines such as: harmine, harmaline (identical with harmidine), harmalol and harman and quina-zoline derivatives: vasicine and vasicinone [4]. It is known that these alkaloids have a wide spectrum of neuropharmacological, psychopharmacological, and antitumor effects.

Therefore, they have been traditionally used in oriental medicine for the treatment of various diseases including cancers [5].

There is great variety of pharmacological and biological activities of *P. harmala* such as antimicrobial, to be effective in the treatment of dermatosis, hypothermic [6]. Recently groups of workers reported that the extract of *P. harmala* possesses antinociceptive analgesic and anti-inflammatory properties [7]. Based on the strength above, this study was proposed aiming to:

1- Detect the antitumor activity of *P. harmala* seeds.
2- Investigate the immunomodulatory effect of *P. harmala* seeds.
3- Determine whether pre- or post-treatment with *P. harmala* is the best against antitumor drugs.

Materials and Methods

Plant Materials

The seeds of *P. harmala* that are suspected to be active compounds containing parts were purchased from the local market of Baghdad governorate.

Extraction of Plants Compounds (Methanolic Extract)

A quantity of (100 g) of *P. harmala* powder was extracted with 500 ml methanol by Soxhelt apparatus for 6 hours at 40-60^{0}C, and then the cooled solution was evaporated to dryness by oven at 40^{0}C. The amount of *P. harmala* powder extract was 12.0 g (12 % of the original weight) and stored in refrigerator at 4^{0}C until used [8].

Animal Grouping and Treatment

Albino male mice, weighting (25-40) g their ages ranging from (30-45) day were used throughout the study. The animals were divided into four groups designated as A, B, C and D. each group consists of 5 animals and treated as following:

Group A: The animals were treated with distilled water (negative control).

Group B: The animals were treated with methotrexate (MTX "Shaanxi China") a dose of 50 mg/kg (positive control).

Group C: this group was subdivided into two subgroups as follow:

C1: These were treated with a dose of 75 mg /kg of the extract.

C2: These were treated with a dose of 100 mg /kg of the extract, followed by treating both subgroups with 50 mg / kg of MTX for pre-treatment study.

Group D: This group was designed to study the Post-treatment activity of *P. harmala* Extract. The animals were treated with a dose of 50 mg / kg of MTX and subdivided into two subgroups as follow:

D1: the animals were treated with 75 mg / kg of the extract.

D2: the animals were treated with 100 mg / kg of the extract.

The tested materials were injected orally using gavage needle as a single dose (0.1 ml) per day for seven days. Then, the mice were sacrificed on day 8 for laboratory assessment.

Laboratory Methods

Cytotoxicity Testing

Cytotoxicity of *P. harmala* was screened on L20B (a mouse tumor cell line used at the passage 15) was obtained from the Biotechnology Research Center/ Al-Nahrain University.

Preparation of Concentrations

The following concentrations were prepared immediately before use by dissolving 10 g in 100 ml of serum free medium (*SFM*) followed by preparing (60.25, 125, 250, 500 ,1000) µg/ml. depending on the LD50 of *P. harmala* which was 425 mg / kg as mentioned by [6]. So the IC50 of this study was 200 µg/ml.

Viable Cell Count

Cells count were performed according to Freshney [9], using trypan blue dye, dead cells take up the dye making them easily distinguished from viable cells. Which counted by Neubauer chamber.

Tumor Inhibition Assay

The procedure below was depending on cytotoxicity testing mentioned in Freshney [9], which was applied for both L20B cell line and chick embryo primary culture.

Cell suspension was well mixed and treated with both extracts poured into 96 flat bottom well microtiter plate and incubated at 37°C for 72hr in an incubator supplemented with (5%) CO_2. 50 µl/well of neutral red dye were added and incubated again for 2 hr. The results were read by ELISA reader at 492 nm.

Total Leucocytes Count

Blood samples were collected by heart puncture using a disposable syringe (1 ml) pre-coated with heparin. The method of Haen [10] was followed, in which, an aliquot of 0.02 ml blood was mixed with 0.38 ml of leucocyte diluents, and examined in Neubauer chamber, and counted using the following equation:

Total count (cells/c.mm.) = (No. of cells counted/4) x 20 x 10

Absolute Count of Leucocytes

One drop of blood was smeared on a clean slide and left to dry at room temperature. The smear was stained with Leishman stain for 5 minutes and buffered for 10 minutes, and then washed with tap water. The slide was air-dried, and then examined under oil immersion lens (100X) [10]. At least 100 leucocytes were examined, and the percentage of each type was recorded, while the total count of each type was obtained using the following equation:

Total count of each type= (percentage of cells X total count / 100)

Phagocytic Index

Phagocytosis was carried out depending on the procedure of Metcalf [11] in which the peritoneal cells were suspended in 1 ml of normal saline, followed by mixing 0.2 ml of cell suspension, 0.1 ml of heat killed yeast suspension and 0.1 ml of human AB plasma and incubated in a shaking water bath at 37°C for 30 and 60 min. then, smears were made and stained with Giemsa stain snd examined under oil immersion lens (100 X). At least 100 yeast-phagocytic activities were expressed as a phagocytic index, which calculated as follow:

Phag. Index (%) = (No. of phag. Cell / Total count) x 100

Statistical Analysis

The results were statistically analyzed to determine the significance effect among the concentrations of both extracts and their effect on tumor cell line and normal cells. The comparison between groups has based on analysis of variance test (ANOVA), while the significance differences based on Duncan's test [12].

Results

Cytotoxicity of *P. harmala*

As shown in table (1), this crude extract of *P. harmala* has cytotoxic effect in the growth of L20B cell line starting at the concentration 125 µg/ml with high significance difference (P≤0.05) accompanied with increased cytotoxic effect toward the higher concentrations when compared with the negative control during this incubation period 72 hr. also, the positive control (MTX) has showed clear cytotoxic effect in the growth of this cell line starting from the lesser concentration (62.5 µg / ml) with high significant effect (P<0.05) in the growth of this cell line at the same incubation period.

Table (1): The cytotoxic effect of *P. harmala* methanolic crud extract on L20B cell line after incubation period for 48 *hr*.

Conc. (µg /ml)	OD of cell conc. ± SE		
	P. harmala	-ve ctrl	+ve ctrl (MTX)
62.5	A,a* 0.481±0.042	A,a 0.487±0.097	A,b 0.279±0.081
125	B,a 0.423±0.033	A,b 0.483±0.028	A,b 0.273±0.062
250	BC,a 0.382±0.065	A,b 0.486±0.056	A,b 0.272±0.056
500	C,a 0.337±0.089	A,b 0.484±0.066	AB,b 0.259±0.072
1000	D,a 0.296±0.085	A,b 0.482±0.047	B,b 0.234±0.022

***Different letters mean significant effect P≤0.05; small letters to compare between columns, capital letters to compare between rows.**

Total and Differential Leucocytes Count

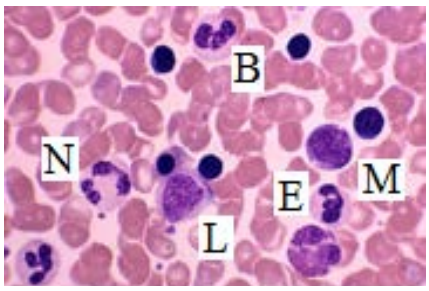
As shown in Table (2), MTX (50 µg/kg) treatment cause significant decrease (P<0.05) in the total counts of leukocytes to reach to (51.40±4.04) as compared with the negative control (103.80±2.86). A significant decrease (P<0.05) in the number of lymphocyte and neutrophils was shown after MTX treatment to reach to (2578±324 and 2293.0±304.5) cell/c.mm. blood respectively in comparison with the negative control (5439±265 and 4420.4±317.2) cell /cu.mm. blood respectively. But regarding monocytes, eosinophil and basophil, treatment with MTX showed no significance effect.

Table (2): The effect of MTX and *P. harmal* methanolic extract on total and differential count of WBC cells.

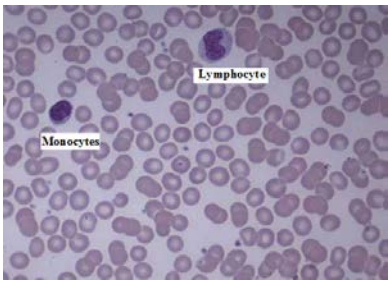
Groups	Total WBCs (m±SE)	Differential count (m±SE)				
		No.of Lymphocyte cells /cu. mm. blood	No.of Neutrophil cells /cu. mm. blood	No.of Monocyte cells /cu. mm. blood	No.of Eosinophil cells /cu. mm. blood	No.of Basophil cells /cu. mm. blood
Negative control	A* 103.80±2.86	A 5439±265	A 4420.4±317.2	A 332.4±88.9	A 167.6±19.18	A 20.40±4.562
Positive control (MTX) (50 µg/kg)	B 51.40±4.04	B 2578±324	B 2293.0±304.5	A 175.4±92.7	A 72.0±5.96	A 21.40±2.941
Pre-treatment 100 mg/kg	C 177.40±3.44	C 9388±959	B 7391.8±207.2	B 639.0±77.3	A 249.8±27.18	A 71.60±9.807
Pre-treatment 75 mg/kg	D 158.20±9.52	D 8486±902	AB 6523.4±229.2	AB 597.2±83.6	A 181.2±24.87	A 31.80±7.111
Post-treatment 100 mg/kg	E 170.27±6.54	CD 8825±677	B 7040.1±195.7	B 750.0±90.3	A 162.3±32.10	A 68.95±9.458
Post-treatment 75 mg/kg	D 157.04±3.31	C 8256±628	AB 6882.3±180.1	AB 377.6±86.1	A 156.5±19.41	A 31.69±7.085

*** Differences A, B, C are significant (P<0.05) in same column.**

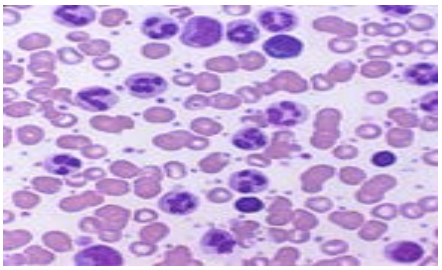
Also, figure (1) shows the reduction and increases in number of leucocytes when examined in the blood film of the tested mouse.



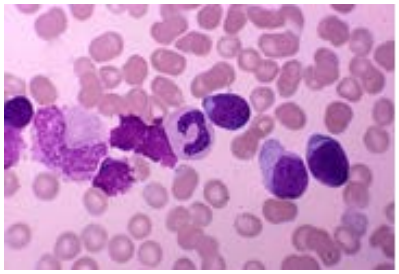
(A) Leucocytes (negative control) 400 X



(B) Leucocytes (MTX treated) 100 X



(C) Leucocytes (Pre-treatment) 100 X



(D) Leucocytes (Post-treatment) 400 X

L: Lymphocyte , M: Monocyte, N: Neutrophil , B: Bsophil , E: Eosinophil

Fig (1): Leucocytes of the tested mice.

Regarding pretreatment by methanolic extract of *P. harmala*, Table (1) shows that the two different concentration of *P. harmala* extracts caused an increase in the total number of leukocyte which reached to (177.40±3.44) for (100 mg/kg) and (158.20±9.52) for the (75 mg/kg) of extract. The increase in total counts was significant (P<0.05) for both concentration in comparison with total counts of negative control (103.80±2.86).

Also there was a significant (P<0.05) increasing in number of both lymphocyte and neutrophil for both concentration of *P. harmala* , the number of lymphocyte reach to (9388±959 and 8486±902) cells/c.mm. blood, for both (100 and 75) mg/kg respectively in comparison with negative control (5439±265) cell/c.mm. blood, and the number of neutrophil reach to (7391.8±207.2 and 6523.4±229.2) cell/c.mm. blood respectively for both concentration in comparison with negative control (4420.4±317.2) cell/c.mm. blood. Also, monocyte count has shown an increase that are reached (639.0±77.3 and 597.2±83.6) for both concentrations (100 and 75) mg/kg respectively but only the concentration (100 mg/kg) has showed significant increase (P<0.05) when compared with the negative control (332.4±88.9).

Regarding eosinophil and basophil, there is no significant increase (P>0.05) when their negative control were (167.6±19.18 and 20.40±4.562) cell/cu.mm. blood respectively; the numbers of eosinophil reached to (249.8±27.18 and 181.2±24.87) cell/cu.mm. blood for both concentration (100 and 75) mg/kg respectively , while the numbers of basophil were (71.60±9.807 and 31.80±7.111).

On the other hand the two different concentrations (100 and 75) mg/kg of *P. harmala* extracts in case of post-treatment, cause significant increase ($P<0.05$) in the total number of WBC to reach to (170.27±6.54 and 157.04±3.31) for both concentrations respectively in comparison with the totals counts of the negative control (103.80±2.86).

Also there is a significant increase ($P<0.05$) in number of lymphocyte and neutrophil for both concentration of *P. harmala* extracts, the number of lymphocyte reached to (8825±677 and 8256±628) cell/c.mm. blood respectively in comparison with negative control (5439±265) cell/c.mm. blood and the number of neutrophil has reached to (7040.1±195.7 and 6882.3±180.1) cell/c.mm. blood respectively in comparison with the negative control (4420.4±317.2) cell.c.mm. blood. Monocytes have shown significant ($P<0.05$) only at the concentration (100 mg /kg) of *P. harmala* extracts at which the count was reached (750.0±90.3) cell/c.mm. blood in comparison with the negative control (332.4±88.9), while at the concentration (75 mg /kg), there was no significant effect when the number of monocyte was (377.6±86.1) cell.c.mm. blood.

As post-treatment with *P. harmala* extracts, both eosinophils and basophils have showed no significance effect for both concentrations when compared with their corresponding negative control. Cytotoxic effect of MTX which cause damage of the cell in addition to its effects on bone marrow (Decreasing in cellular division), this damage expected to possess an effects on the quantity of WBCs.

Phagocytic Index

The results explained in table (3) showed that the cells treated with MTX (positive control) possessed decrease (1.08±0.025 %) in phygocytic index of the phagocytic cells with significance ($P>0.05$) when compared with the negative control (1.22±0.037 %).

At the same time, there is a significance increase ($P<0.05$) in phagocytic index of the animals that are pre- and post-treated with the methanolic extract of *P. harmala* when compared with the negative control (1.22±0.037 %).

In pre-treatment, the concentration (100 µg/kg) possessed phagocytic index (2.7±0.073 %), while the concentration (75 mg/kg) has effect reached to (2.38±0.071 %). On the other hand, in post-treatment both these concentrations have an increased values of phagocytic index which were (1.55±0.065 and 1.11±0.062) % respectively.

Table (3): The effect of MTX and *P. harmala* methanolic extract on mouse phagocytic cell.

Groups		Phagocytic Index % (m+SE)
Negative control		B 1.22±0.037
Positive control (MTX) (50 µg/kg)		A 1.08±0.025
Pre-treatment	100 mg/kg	C 2.7±0.073
	75 mg/kg	D 2.38±0.071
Post-treatment	100 mg/kg	D 1.55±0.065
	75 mg/kg	C 1.11±0.062

*** Differences A, B, C and D are significant ($P<0.05$) to compression column**

The purpose of phagocytic index is to explain the role of our extracts in activation of innate immune response. Figure (2) explains the activity of phagocytic cells of treated mouse in phagocytosis of heat killed yeast.

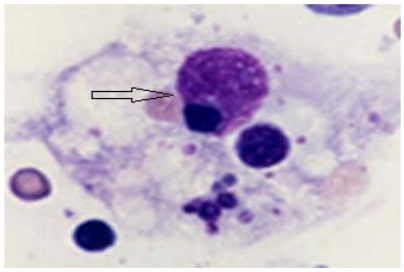


Fig (2): Phagocytic cell opsonize heat killed yeast (1000 X).

Discussion

The objective of this research is to evaluate the antitumor activity of *P. harmala* in addition to their ability to suppress the side effect resulted from treatment with antitumor drugs.

The present study indicated that the extracts of *P. harmala* had remarkable antitumor activity in L20B cell line. These crude extracts exert obvious antitumor potential on L20B cell line and these reasonable results since this plant rich with many active compounds that are potentially inhibit the tumors.

Many naturally occurring alkaloids (e.g., camptothecin, vincristine and ellipticine) are potent anticancer agents, These alkaloids (which are the main alkaloids present in *P. harmala*) kill tumor cells by different biochemical modes of action, such as inhibition of apoptosis, inhibition of topoisomerase I and II and inhibition of microtubule formation [3]. While [13] has demonstrated that harmine and its derivatives play a significant role in up-regulating CD95, a well-known death receptor that is triggered by its cognate ligand or agonistic antibody. The molecular mechanisms for the antitumor activity of β-carbolines are not fully defined, but induction of apoptosis, inhibition of DNA topoisomerase I and II and CDKs may be implicated [14]. On the same side, β-carbolines and its derivatives downregulated Bcl-2 Members family proteins which are key regulators of apoptosis by acting on mitochondria; this protein family includes both anti-apoptotic molecules such as Bcl-2 and Bcl-X(L), and proapoptotic molecules [15].

On the other hand, the tumor-killing activity of these compounds may involve both “intrinsic” and “extrinsic” apoptotic pathways that can lead to the activation of effector caspases; Harmol induced caspase-3, caspase-8, and caspase-9 activities and caspase-3 activities accompanied by cleavage of poly-(ADP-ribose)-polymerase [16]. Furthermore, harmol treatment induces the release of cytochrome c from mitochondria to cytosol; Cytochrome *c* interacts with Apaf-1, dATP/ATP and procaspase-9 to form apoptosome, which results in autocatalytic processing of caspase-9 and initiation of the caspase cascade, which completely inhibit apoptosis [17]. Also, harmaline can induce necrosis which could result in the intracellular release of degrading enzymes from subcellular particles such as lysosomes, which may cause partial digestion of DNA [18].

Immunological Parameters

A decreasing in total counts of leukocyte may be returned to cytotoxic effects of MTX; the decreasing in number of monocyte is thought to be that MTX cause reduction in monocytic IL-1 production, others have suggested that alterations in IL-1 responses were related to diminution in the ability of cells to respond to IL-1 rather than to direct inhibition of its production [19]. The increase in number of leucocytes may be attributed to the inflammatory response induced by these extracts that possess a wide range of anti-inflammatory activities [6]. These results will agreement with [22] who found that this cell will increase in breast cancer patients and in albino female rats after treatment with tamoxifen drug. These results lead to detect the role of these extracts in both adaptive immune response represented by lymphocytes and innate immune response represented by neutrophils [23].

These effects may be attributed to the active compounds present in flavonoids that possess the activity to induce innate immune response like inflammation and monocyte reproduction [24]. While [24] have pointed out to the role of flavonoids in increasing the count of WBC making the animal to possess antimicrobial activity.

The results explained in table (3), treating with MTX showed a decrease in phagocytic index (1.08±0.025 %) with a significance variations ($P<0.05$) when compared with the negative control (1.22±0.037 %), this may be attributed to the activity of MTX to inhibit the production of chemotactic factor and macrophage activating factor that are both produced from phagocytic cells [25]. These results may be attributed to the ability of these extracts to support the production of IL-3 and polymorphoneuclear cells and monocytes that act as phagocytic cells, or by the activation of chemotactic factors [27]. Also, these extracts have many substances that stimulate the production of GRD which are known to interact with β_2 integrins, a family of eukayotic adhesion molecules which are constitutively expressed on macrophages [28]. While [24] has showed that the flavonoids have a role in this process when promote the production of monocytes that act as phagocytic cells.

Conclusions

1. *Peganum harmala* crude extracts possess obvious antitumor activity.
2. There were sever side effect on immune response after treatment with MTX and the crude extracts of *P. harmala* suppressed these side effect.
3. The pre-treatment is preferred on post-treatment with *P. harmala* to suppress the side effect of antitumor drugs.

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