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Effects of Crude Alkaloids Isolated from *Peganum harmala* Seeds on the Growth and Metabolism of *Leishmania tropica* Promastigotes

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ABSTRACT

The present work aimed to examine the efficacy of *Peganum harmala* Linn. alkaloids as potential antilieshmanial agents *in vitro*, and to determine their toxicity in mice.

The study included extraction and isolation of *Peganum harmala* crude alkaloids from seeds. The isolated fraction that contains alkaloids was detected, using Myer`s and modified Dragendorff`s reagents. Then, the crude alkaloids were tested for their antileishmanial activity against *Leishmania tropica* promastigotes *in vitro* including their effects on parasite growth and metabolism.

It was found that the studied alkaloids inhibited growth of the parasite remarkably. The inhibitory concentration of 50% of the promastigotes ($IC_{50)}$ at the log phase (96) hrs was 50 µg of the alkaloids/ ml of culture.

Furthermore, the extracted alkaloidal fraction from *Peganum harmala* seeds, resulted in decline of RNA, DNA, and protein content of the parasite and reduced specific activity of dihydrofolate reductase and thymidine phosphorelase enzymes. It also had obvious inhibitory effects on energy metabolism of the parasite.

Oral median lethal dose (oral LD_{50}) of the extracted alkaloids was 1070 mg / kg body weight in Balb/c mice, using the up-and-down method**.**

It can be concluded that *Peganum harmala* alkaloids show promising antilieshmaneal activity and may have potential role in the search for novel antilieshmaneal drugs, as they affect metabolism of proteins, nucleic acids and energy of the parasite (*in vitro)* with a slight toxicity in mice (*in vivo*).

Keywords: *Lieshmania tropica*, *Peganum harmala*, alkaloids, growth, carbohydrates, energy, fumarate reductase, succinate dehydrogenase, dihydrofolate reductase, thymedine phosphorelase, LD_{50}.

تأثير القلويدات الخام المعزولة من بذور الحرمل *Peganum harmala* في نمو وايض أمامي السوط لطفيليات اللشمانيا الجلدية *Leishmania tropica*

الملخص

يهدف العمل الحالي إلى بيان كفاءة قلويدات نبات الحرمل *Peganum harmala* (العائلة الزيتونية) بوصفها مضادات لطفيلي اللشمانيا في الزجاج، ثم تحديد سمية هذه القلويدات في الحيوانات المختبرية.

أستخصلت القلويدات الخام لبذور النبات. أستخدم كاشف ماير وكاشف درانكدروف المحور للتحقق من القلويدات المستخلصة. قدرت الفعالية المضادة للشمانيا للقلويدات المستخلصة في الزجاج *in vitro* باستخدام أمامي السوط للشمانيا الجلدية Leishmania tropica كما قدر تأثير هذه المستخلصات على نمو وأيض الطفيلي.

تبين أن القلويدات قيد الدراسة أثرت على نمو الطفيلي بشكل واضح، التركيز القاتل ل50% من أمامي السوط عند الطور اللوغارتمي (96 ساعة) كان 50 مايكرو غرام من القلويدات/ سم^3 من المزرعة، فضلا عن تسبب الجزء القلويدي المستخلص في انخفاض محتوى الحامض النووي الرايبوزي RNA والرايبوزي منقوص الأوكسجين DNA والبروتين. وكذلك تسببت في اختزال الفعاليةالنوعية للأنزيمين دايهايدروفوليت ردكتيز وثايمدين فوسفورليز, فضلا عن تأثيرها التثبيطي الواضح لأيض الطاقة في الطفيلي.

الجرعة المميتة الوسطية الفموية كانت 1070 ملغم/كيلوغرام من وزن الجسم في الفئران المختبرية سلالة Balb/ c بطريقة الصعود والنزول.

نختم بالقول أن قلويدات الحرمل أعطت فعالية واعدة كمضادات للشمانيا، ويمكن العمل عليها كتراكيب جديدة في مجال بحوث الأدوية المضادة للشمانيا، باعتبار أنها أثرت في أيض البروتين والأحماض النووية والطاقة للطفيلي في الزجاج مع سمية قليلة في الحيوانات المختبرية.

الكلمات الدالة: اللشمانيا الجلدية، الحرمل، قلويدات، نمو، كاربوهيدرات، طاقة، فيوماريت ردكتيز، سكسنيت دايهايدروجينيز، دايهايدروفوليت ردكتيز، ثايمدين فوسفورليز، التركيز القاتل ل50%.

INTRODUCTION

Leishmaniasis is a global parasitic infection, which is prevalent in 88 countries from Tropical to Mediterranean regions, where 12 million people are infected and approximately 350 million people are at risk, with 1-2 million new cases registered annually (WHO,2001). It is a vector-borne disease transmitted by sand flies and caused by an obligate intracellular protozoa of the genus *Leishmania*. There are four major species of genus *Leishmania*, similar in morphology, but different in cultural characteristics, clinical manifestations, geographic distribution, and sand fly vectors, cause disease in humans. *Leishmania tropica*

of Old World cutaneous leishmaniasis or oriental sore is prevalent in some of the Middle-Eastern countries, Tropical Africa and in Asia (including Iraq) (WHO, 2002).

The drugs available for the treatment of leishmaniasis are, in general, toxic, expensive and require long-term treatment. Large-scale clinical resistance against the most commonly used antimonial agents, has been reported (Berman, 1997). Drug treatment of leishmaniasis is complicated by the variation in sensitivity of *Leishmania* species, the different disease manifestations, the lack of controlled clinical trails of new (and old) drugs for leishmaniasis and recently, the increasing levels of antimonial resistance (Croft, 2001). The spread of drug resistance, combined with other shortcomings of the available antilieshmanial drugs (Eibl, 2000), emphasize the importance of the development of new effective and safe drugs against leishmaniasis.

Alkaloids are a large group of compounds that occur throughout the plant kingdom, those have pharmacological important, quinolinic, isoquinolinic, steroidal and β-carboline alkaloids of plant source proved to have a significant antilieshmanial activity against *Leishmania, in vitro* and *in vivo*. (Chan-Bacab and Pena-Rodrigues, 2001). The plant *Peganum harmala* is a source of these compounds (Kamel *et al*., 1970).

Several different protozoan infections have been shown to be susceptible to *Peganum harmala* extracts in varying degrees, including *Theileria s*pp., *Babesia s*pp., *Anaplasma cabali* and *Lieshmania donovani* (Lala *et al*., 2004 ; Mirzaei, 2007). The present work is focused on isolation of crude alkaloids from the seeds of *Peganum harmala* Linn. to determine their toxicity *in vivo* and test their antimetabolic activity of *Leishmania tropica* promastigotes *in vitro*, in addition to their effects on protein, nucleic acids and energy metabolism in the parasite.

MATERIALS AND METHODS

Isolation of *Peganum harmala* crude alkaloids

Seeds of *Peganum harmala* were collected from local markets in Mosul city and taxonomically identified by the botanists Mr. Salim AL-Sufaji, in herbarium of Biology department /College of Science /University of Mosul. The seeds were milled into coarse powder, then extracted with ethanolic alcohol 80% that contains diluted HCL (1N); pigments and unwanted materials were removed by shaking with chloroform. The free alkaloids were then precipitated by the addition of excess ammonia and separated by filtration (Evans, 1997). The extracted fraction which contains alkaloids was detected, using Myer`s reagent (Potassiomercuric iodide solution) (Sousek *et al.,* 1999) and modified Dragendorff`s reagent (Potassium bismuth solution) (AL-Shahaat, 1986).

Biological activity of the extracted seeds alkaloids *in vitro*

A. *Leishmania* used:

MHOM / IQ / 1992 / MREC3 *Leishmania tropica* stock culture was used. The culture was obtained from the College of Medicine AL-Nahreen University, which has been characterized using isoenzyme method according to AL-Jeboori and Evans (1980). The obtained culture was cultivated in Tobie`s medium (Tobie *et al*., 1950).

B. Cultivation and estimating numbers of parasites:

1.9 ml of liquid phase was added to McCantry vialscontaining 5 ml solid phase slants, 0.1 ml of *Leishmania* promastigotes inoculums was taken from stock culture during logarithmic phase, so that the initial density of the organism was 2 x 10^5 / ml, then the number of organisms of new culture incubated at 26 C^o for 4 days, was counted directly using a haemocytometer.

C. Effect of the extracted *Peganum harmala* seeds alkaloids on growth, generation number and generation time of *Leishmania tropica* promastigotes:

Effect of the isolated alkaloids on growth was studied *in vitro* in comparison with untreated groups. The compound was dissolved in 2% dimethyl sulfoxide (DMSO). Six concentrations (20,30 , 40 , 50 , 60 , 70) µg / ml were prepared to determine the IC_{50} and IC_{90} of the cultivated organisms. Numbers of promastigotes were determined at different time intervals (24 , 48 , 72 , 96) hours, then generation number and time of each culture at each time interval was estimated using Benjamin and German laws (1993).

D- Biochemical studies:

a- Estimation of total proteins, nucleic acids, and carbohydrates:

Lowry *et al*., (1951) method was used for protein quantitative determination. While Schneider's method (1957) was used for quantitative estimation of total nuliec acids, DNA, and RNA. Herbert *et al*., (1971) method were carried out to estimate total carbohydrates in treated and untreated parasites.

b-Enzyme studies:

Determination of **thymidine phosphorelase** (TP) activity was carried out using an assay system, which has been adopted by AL-Janker (1999). Activities were measured by assaying one of the products, deoxyribose-1-phosphate, using the diphenylamine method (Burton, 1956). Specific activity of TP was expressed as the number of nanomoles of produced deoxyribose-1-phosphate. Absorbance was measured at 600 nm spectroscopically.

Dihydrofolate reductase (DHFR) activity was determined depending on assay system, which was illustrated by Baccnari *et al*., (1975) and adopted by Al-Chalabi and AL-Khayat (1986). Unit of enzyme activity was expressed as the amount of enzyme required for oxidation of 1 µM of NADPH/minute of reaction depending on NADPH extinction coefficient =6.2 × 103 M-1cm-1, Absorbance was measured at 340 nm (Mathews *et al*., 1963).

Succinate dehydrogenase (SDH) activity was determined using method adopted by Al-Hasany (2004). It depends on reduction of the co-enzyme, flavine adenine dinucleutide (FAD) To FADH2 by succinic acid (SA):

Succinic acid + SDH-FAD ⟷ Fumaric acid +FADH2

Specific activity of succinate dehydrogenase = number of FAD reduced nanomoles / minute/ mg protein, Absorbance measured at 720nm.

Activity of **NADH-depending fumarate reductase**(FRD- NADH) enzyme was assessed by a modified method originally described by Denicola-Seoane *et al*., (1992) and adopted by Chen *et al*., (2001). Unit of enzyme activity was expressed as the amount of enzyme required for oxidation of 1 µM of NADH/minute of reaction depending on NADH extinction coefficient =6.2 × 10^3 M-1cm^{-1} and absorbance of 340nm.

Determination of the median lethal dose (LD_{50}) of the isolated crude alkaloids in mice

Oral medium lethal dose of *Peganum harmala* crude alkaloids in Balb/c mice were determined using up-and-down method (Dixon, 1980). Male mice aged 4 – 6 weeks were injected with different doses of the isolated alkaloids. Primary dose (500 mg / kg body weight) was chosen after conducting series of test levels.

Statistical analysis used in this study included: F-test using analysis of variance (ANOVA table) and Duncan's multiple range test and difference between two samples mean (t-test). The level of significance was $P \leq 0.05$ (AL-Rawi and Kalaf-Allah, 1980 ; Daniel *et al.*, 1983).

RESULTS AND DISCUSSION

Therapeutic evaluations for medicinal plants are essential because of the growing interests in alternative therapies and the use of natural products. Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans (Wright and Phillipson, 1990).

The pharmacologically active compounds of *Peganum harmala* are several alkaloids, which are found especially in the seeds and the roots. These include β-carbonile alkaloids such as harmine, harmaline, harmalol and harman, and quinazoline alkaloides such as vasicine and vasicinone (Kamel *et al*., 1970). Thus, In the present work, crude alkaloids of *peganum harmala* seeds where isolated in the form of brown colored sticky material. As shown from alkaloids detection, which was accomplished using Myer's test (Sousek *et al.*, 1999) and modified Dragendorff's test (AL-Shahaat, 1986). Results were positive with both tests; I e. Production of creamy color precipitate and reddish-brown precipitate, respectively.

1. Effect of isolated crude alkaloids on the growth, generation number and generation time of *Leishmania tropica* promastigotes *in vitro*:

Crude alkaloids isolated from *Peganum harmala* seeds were tested for their antileishmanial properties *in vitro*. Table 1 shows the inhibitory effects of different concentrations of the crude alkaloids on *Leishmania* growth in comparison with control group, during different time intervals. Relatively all concentrations used demonstrated significant inhibitory effects against *Leishmania* promastigotes. An inverse relationship could be established between concentration of the alkaloids and growth mean of the parasite. Inhibitory concentration of 50% of promastigotes (IC_{50}) was 50 µg / ml, where as IC_{90} was 70 µg / ml at logarithmic phase (96hrs. of cultivation).

On the light of the growth indices (Table 1), effect of the crude alkaloids on generation number and generation time of *Leishmania tropica* promastigotes were estimated (see paragraph C of materials and methods). As for the effect of different concentrations of the extracted alkaloids on generation number (Table 2), inverse correlation between generation number and concentration were observed, generation number at log phase ranged from 6.18 generations at 20 µg alkaloids / ml culture to 4.08 generations at 70 µg / ml, when compared with control group (6.74 generations).

However, generation time appeared to depend upon the concentration of the alkaloids (Table 3). Generation time increased when concentration increased (direct correlation). At

log phase, generation time values ranged from 15.53 hrs. at 20 µg / ml to 19.35 hrs. at 70 µg / ml when compared with control group (14.24) hrs.

The significant inhibitory effect of *Peganum harmala* alkaloids on the growth of *Leishmania tropica* promastigotes may be elucidated by different reasons. Mirzaie (2007) pointed out that the IC_{50} of *Peganum harmala* acidic extract was 1832.65 µg / ml of *Lieshmania major* growth. Lala *et al*., (2004) estimated that the death of *Leishmania donovani* promastigotes that were pretreated with harmine alkaloids could be attributed to cell necrosis due to non-specific membrane damage. On the other hand, Li *et al*., (2007) revealed that, some beta-carboline alkaloids, including some *Peganum harmala* alkaloids, are specific inhibitors of cyclin dependent kinases (CDKs). Therefore, to give a picture on the mode of action of *Peganum harmala* alkaloids, as antilieshmaneal agents at molecular level, further investigation applied using biochemical tests in the next part of the research is required.

Table 1: Effect of different concentrations of the isolated alkaloids on numbers of *Leishmania tropica* promastigotes at different time intervals.

Exposure time (hrs.) / Treatments (µ g / ml)	24	% inhibition	48	% inhibition	72	% inhibition	96	% inhibition
	Mean* ± SE		Mean* ± SE		Mean* ± SE		Mean* ± SE	
Control	14.67 ± 0.26 d	--	29.50 ± 1.60 e	--	122.00 ± 3.33 f	--	210.33 ± 6.39 e	0
20	11.00 ± 0.76 c	25	25.00 ± 2.08d	15	86.50 ± 5.31 e	29	143..02± 4.28 d	32
30	9.17 ± 0.44 b	38	22.00 ± 1.52 c	25	76.86 ± 0.23 d	37	136.71 ± 0.04 d	35
40	8.17 ± 0.44 bc	44	20.67 ± 0.93 c	30	73.67 ± 0.75 d	40	118.50 ± 4.07 c	41
50	7.17 ± 0.60 b	51	14.83 ± 0.72 b	50	59.33 ± 2.44 c	51	102.33 ± 2.80 b	49
60	4.00 ± 0.29a	73	9.50 ± 0.30 a	68	26.33 ± 0.73 b	78	50.83 ± 2.20 a	75
70	2.67 ± 0.33a	81	9.00 ± 0.03a	69	11.33 ± 0.60a	91	33.50 ± 2.84a	83

* Three replicates were used for each treatment. Mean and Standard Error were multiplied x 10^5.

** Initial number of promastigotes used in each culture = 2 x 10^5.

*** Different letters refers to presence of significant differences between treatments at $P \leq 0.05$, according to Duncan's test.

Table 2: Effect of different concentrations of the isolated alkaloids on generation number of *Leishmania tropica* promastigotes at different time intervals.

Exposure time (hrs.) / Treatments (µ g / ml)	24	48	72	96
	Mean* ± SE	**Mean* ± SE**	**Mean* ± SE**	**Mean* ± SE**
Control	2.9 ± 0.04 ^{e}	3.90 ± 0.26 ^{d}	5.95± 1.50 ^{e}	6.74 ± 0.11 ^{d}
20	2.47 ± 0.09 ^{de}	3.66 ± 0.00 ^{cd}	5.45 ± 0.22 ^{d}	6.18 ± 0.12 ^{c}
30	2.2 ± 0.00 ^{d}	3.47 ± 0.06 ^{c}	5.28 ± 0.11 ^{d}	6.11 ± 0.003 ^{c}
40	2.04 ± 0.02 ^{d}	3.38 ± 0.05 ^{c}	5.22 ± 0.23 ^{d}	5.91 ± 0.46 ^{b}
50	1.85± 0.01 ^{c}	2.90 ± 0.16 ^{b}	4.91 ± 0.04 ^{c}	5.70 ± 0.60 ^{b}
60	1.01 ± 0.01 ^{b}	2.26 ± 0.05 ^{a}	3.73 ± 0.01 ^{b}	4.96 ± 0.03 ^{a}
70	0.42± 0.01^{a}	2.18 ± 0.01^{a}	2.51± 0.01^{a}	4.08± 0.02^{a}

Table 3: Effect of different concentrations of the isolated alkaloids on generation time (hours) of *Leishmania tropica* promastigotes at different time intervals.

Exposure time (hrs.) / Treatments (µ g / ml)	24	48	72	96
	Mean* ± SE	**Mean* ± SE**	**Mean* ± SE**	**Mean* ± SE**
Control	8.28 ± 0.82 ^{a}	12.30 ± 0.31 ^{a}	12.10 ± 0.04 ^{a}	14.24 ± 0.17 ^{a}
20	9.72 ± 2.11 ^{b}	13.11 ± 0.00 ^{b}	13.21 ± 0.90 ^{b}	15.53 ± 0.19 ^{b}
30	10.91 ± 0.00 ^{c}	13.83 ± 0.81 ^{bc}	13.64 ± 0.83 ^{c}	15.71 ± 0.01 ^{b}
40	11.76 ± 1.84 ^{d}	14.20 ± 0.60 ^{c}	13.79 ± 1.22 ^{c}	16.24 ± 1.33 ^{cd}
50	12.97± 2.35 ^{e}	16.55 ± 0.28 ^{d}	14.66 ± 0.15 ^{d}	16.84 ± 1.88 ^{d}
60	23.76 ± 4.36 ^{f}	21.24 ± 1.73 ^{e}	19.30± 0.11 ^{e}	19.35± 1.89 ^{e}
70	57.14 ± 2.02 ^{g}	22.02 ± 1.08 ^{f}	19.30± 0.11^{f}	19.35± 1.89 ^{f}

2 - Biochemical studies:

I - Effect of IC_{50} of *Peganum harmala* crude alkaloids on total protein, nucleic acids content and some related enzymes of *Leishmania* promastegotes *in vitro*:

Peganum harmala alkaloids found to cause significant reduction in protein, DNA and RNA content in *Leishmania tropica* promastigotes that were treated with IC_{50} of the tested alkaloids (Table 4), the higher reduction caused by these alkaloids was obsereved on the percentage concentration of DNA (41.82%). In the present work, and on the light of efficacy of *Peganum harmala* alkaloids as protein and nucleic acids metabolic inhibitors, the specific activity of thymidine phosphorelase (TP) and dihydrofolate reductase (DHFR) of the treated parasites were estimated. The isolated alkaloids found to reduce 42.59% of thymidine phosphorelase (TP) specific activity and 26.23% of dihydrofolate reductase (DHFR) specific activity (Table 5). Furthermore, it should be taken into account that particular antiparasitic agent may affect metabolism of protein when affects nucleic acids metabolism of the parasite.

Proteins of protozoan cells have similar characters of those in higher organism (Bryent and Behm, 1989). Many antiprotozoan agents affect metabolism of proteins and nucleic

acids of the target organism like ketokonazole, allopurinol, chloropromazine, ethidium, dihudroemitine and soramine (Hassan, 1979 ; AL-Healy, 2000), and different modes of action suggested for antilieshmanial drugs as protein and nucleic acids metabolic inhibitors (Hassan, 1979; Chang *et al*., 1985; AL-Chalabi, 1986 ; AL-Jebouri, 2002).

On the other hand, beta-carboline alkaloids present in medicinal plants, such as *Peganum harmala* and *Eurycoma longifolia*, have recently drawn attention due to their antitumor activities. Further mechanistic studies indicate that beta-carboline derivatives inhibit DNA topoisomerases in yeast and then interfere with DNA synthesis (Li *et al.,* 2007). Besides, peganine hydrochloride dehydrate (isolated from *Peganum harmala* seeds) found to inhibit DNA topoisomerase, thus interfere with DNA synthesis in *Lieshmania donovani* promastigotes and amastigote (Misra *et al*., 2008). Li *et al*., (2007) revealed that, some beta-carboline alkaloids (such as *Peganum harmala* alkaloids) are specific inhibitors of cyclin dependent kinesis (CDKs). They concluded that the antitumor activity of beta-carboline drugs could be attributable to their inhibition of CDK.

Because thymidine nucleotides are important precursors for DNA synthesis, a number of enzymes involved in its metabolism has been investigated in *Leishmania*. In addition, *Leishmania* and *Trypanosoma* lack a significant salvage pathway for dTMP (Hassan, 1979). These properties have important implications for rational chemotherapy of leishmaniasis, and since in many cells; any strategy aimed at dTMP deprivation would require the simultaneous blockage of both *de novo* and *salvage* anabolic pathways. Therefore, effects of the isolated alkaloids on the activity of DHFR and TP were taken into account to point out the possible mechanism of action of these alkaloids as nucleic acids and polypeptides synthesis inhibitors in leishmania parasite.

Results illustrated in Table (5) showed that the tested alkaloids had significant effects on dTMP synthesis, since they affect activity of DHFR, a key enzyme in the synthesis of thymidylate by *de novo* pathway, and therefore of DNA(Chang *et al.,* 1985). On the other hand, they showed significant inhibitory effects on TP activity, the important enzyme in the synthesis of dTMP by salvage pathway in *Leishmania* promastigotes (Chang *et al*., 1985), i.e. the former results indicated that the tested alkaloids had relatively significant inhibitory effects on both *de novo* and *salvage* pathways in the parasite. These results reflected in a way the high inhibitory effects of these alkaloids on DNA synthesis, i.e. DNA content (see Table 4). AL-Chalabi (1986) demonstrated that treating *Leishmania tropica* promastigotes with 10-4mol/ml of paramomycine sulfate inhibited 50% of thymidylate syanthetase activity; he concluded that this effect may result in interaction with DNA synthesis.

DHFR inhibitors have been used in chemotherapy of malaria for nearly 40 years (Fernando and Dolores, 2001). It is also a potential target for developing drugs against leishmania species and trypanosomes (Zuccotto *et al*., 1998). The bases of the antiinfective selectivity of folate antagonists, like trimethoprim and pyrimethamine and methotrexate, are potent inhibitors of bacteria and protozoal DHFRs. These species of selective agents apparently exploit the differences in the active site regions of the parasite and host enzymes (Schweitzer *et al*., 1990). AL-Khayat (1981) found that treating *Leishmania donovani* and *Leishamnai tropica* promastigotes with aminoptirine and methotrexate reduced 50% of DHFR activity; she also found that some antifolate compounds like methotrexate have high affinity to compete with folate and inhibit DHFR activity; as a result, they inhibit the synthesis of dTMP.

Furthermore, some plant extracts that contain alkaloids have been found to have inhibitory effects on dTMP metabolism, for example, AL-Khan (2001), revealed that aqueous extract of *Capparis spinosa* increased activity of *Leishmania major* TP about 20.6%, and decreased thymidylate syanthetase activity of the same parasite about 17.6%. He concluded that salvage pathway takes the place of *de novo* pathway and this and this explain the increase of TP activity in opposite to decrease of thymidylate syanthetase activity.

AL-Jubouri (2005) revealed that treating *Leishmania tropica* promastigotes with IC_{50} of *Nirium oleander* extract reduced 73% of DHFR activity. AL-Fahhady (2006) demonstrated that treating *Leishmania tropica* promastigotes with IC_{50} of a *Urtica piluifera* reduced 28.9% of DHFR activity. It could be concluded that dTMP is apossible site of action for the isolated alkaloids as antilieshmanial agents.

II- Effect of IC_{50} of *Peganum harmala* crude alkaloids on energy metabolism (carbohydrate content and some related enzymes) of *Leishmania* promastegotes *in vitro*:

Table 6 shows that there is an increase in glucose (CHO) content in parasites those treated with IC_{50} of the tested alkaloids when compared with control group. As its known, carbohydrates are important source of energy in living organisms including parasites (Bryant and Behm, 1989), i.e. carbohydrates residue (17.94%) in treated parasite may indicate energy metabolism defect. Thus, specific activity of two enzymes (succinate dehydrogenase and NADH depending fumarate reductase) was reported in respiratory chain of *Leishmania* parasite (Hellemond and Tielens, 1997).

Table 7 shows that the tested alkaloids decrease significantly both the specific activity of fumarate reductase and succinate dehydrogenase, thus affect cellular respiration in the parasite. This result somewhat explains the accumulation of glucose in treated parasites. Thus, the two mitochondrial enzymes, FRD-NADH and SDH, of *Leishmania tropica* promastigotes were proved to be the site of action of the isolated alkaloids, i.e. they are involved in the inhibition of parasites energy metabolism, and, since, the inhibitory effect of the experimented alkaloids against parasite SDH activity was 11% higher than that on inhibitory effect of these alkaloids against FRD-NADH activity (Table 7), leishmania SDH is more sensitive to the tested alkaloids than FRD-NADH. These data my indicate the inhibitory effects of these alkaloids on respiratory enzyme (energy metabolism) of both mammalian host and the parasitic cells. This result may refer to the possible toxicity of *Peganum harmala* alkaloids to mammalian animals.

Some well-known antiprotozoal drugs and some potential antiprotozoal compounds influence the energy metabolism of leishmania, like pentamidine isothionate core, sodium stibogluconate (Berman *et al*., 1989) and mycotoxin MT 81 (Majumdar *et al.,* 1993). Misraei (2007) Found that the alkaloid compound vasicine (peganine) had significant antileishmanial activity against promastigote stage of *Leishmania donovani*. Which induce apoptosis in *L. donovani* amastigote and promastigotes via loss of mitochondrial transmembrane potential.

As it is known, biological system of energy metabolism is essential for the survival, continued growth and reproduction of the living organisms. Typical mitochondria are usually considered to be oxygen-consuming, ATP-producing organelles. They use pyruvate

dehydrogenase for oxidative decarboxilation of pyruvate to acetyl coenzyme A, which is then completely oxidized to CO_2 through the Krebs cycle. (Champ *et al*., 2005).

In parasitic organisms, the carbohydrate and energy metabolism differ greatly from those of their vertebrate hosts. The most important factors in this respect are the nutrient and oxygen supply (Bryant and Behm, 1989). For example leishmania promastigotes depend mainly on respiratory chain activity for energy generation, have a poor capacity for anaerobic functioning, and go into a reversible metabolic arrest during anoxia, which enables them to survive anoxia for at least 48 hours (Santhamma and Bhaduri, 1995). Hellemond and Tielens (1997) demonstrated that respiratory chain inhibition or anoxia in *Leishmania infantum* promastigotes resulted in a strong inhibition of motility, proliferation, glucose catabolism, in addition to succinate production. This shows that succinate production does not occur via fumarate reduction and cannot be used as electron-sink. The same researchers concluded that leishmania promastigotes lack a true fermentative energy metabolism, in contrast to insect stages of other kinetoplastidae, like Trypanosomes.

FRD catalyzes the reduction of fumarate to succinate, which is a key enzyme in anaerobic energy metabolism for many organisms respiring with fumarate as a terminal electron acceptor. This enzyme has been found among some bacteria such as *Helicobacter pylori* and *Escherichia coli* (Iverson *et al*., 1999), and among protozoal parasites of the genera trypanosoma, plasmodium, and leishmania (see Santhamm and Bhaduri, 1995), and in helminths (Bryant and Behm, 1989).

Fumarate-NADH reductase is obligatory component of the respiratory chain of *Leishmania* parasite, which was shown to be totally absent in mammalian host (Santhamma and Bhaduri, 1995). Because of that, FRD-NADH has been used specially as a target of leishmaniasis treatment by some researchers (Kharmazmi *et al*., 1997). In the present work, effect of the *Peganum harmala* alkaloids on specific activity of promastigotes succinate dehydrogenase was also investigated on FRD-like enzymes in mammalian cells. SDH plays a very important role in the respiratory chains of both mammalian cells and leishmania cell, as one of the classical electron transport enzymes. SDH also conducts the reverse reaction to that which FRD conducts (Hellemond and Tielens, 1997).

In eukaryotes and prokaryotes , aerobically respirated:

Succinic acid + SDH-FAD $\longleftrightarrow$ Fumaric acid +FADH2

In low eukaryotes and prokaryotes under anoxia conditiones:

Fumaric acid +FRD-NADH $\longrightarrow$ Succinic acid + NAD+ 2H

The two proteins, FRD-NADH and SDH exhibit substantial similarities in amino acid sequence, cofactor composition, and mechanism. Indeed, under certain conditions, one enzyme can functionally replace the other and support organism growth (Pealing *et al*., 1992). Because of the central role of fumarate reductase and succinate dehydrogenase in respiration, mutations in this complex can have substantial metabolic consequences. In bacteria, mutations in fumarate reductase can significantly retard growth under appropriate conditions. In higher organisms, mutations of succinate dehydrogenase have been linked to oxidative stress (Iverson *et al*., 1999). In the present work, the effect of *Peganum harmala* alkaloids had no selective effect on *Leishmania* FRD-NADH, thus, this enzyme could not be considered as a target site of antileishmanial activity of these alkaloids.

Table 4: Effect of IC_{50} (50 µg/ml) of *Peganum harmala* alkaloids on the amount of total protein (µg/ml) and total nucleic acids (µg/ml) at log-phase (96 hrs).

Macromolecule type	Macromolecule content µg/ml Mean* ± SE.	%Macromolecule	%reduction
Protein: Control	556.20 ± 8.9	100	0
50 µg/ml	422.22 ± 5.1	75.91	24.09
Total nucleic acids Control	121.55 ± 0.95	100	0
50 µg/ml	91.07 ± 1.2	74.92	25.08
DNA: Control	28.55 ± 0.86	100	0
50 µg/ml	16.61 ±0.35	58.17	41.82
RNA: Control	93.81 ± 1.2	100	0
50 µg/ml	73.77 ± 0.89	78.64	21.36

Table 5: Effects of IC_{50} (50 µg/ml) of *Peganum harmala* alkaloids on the specific activity of Thymidine phosphorelase (TP) and dihydrofolatereductase (DHFR) at log-phase (96 hrs).

Enzyme	Specific activity** Mean* ± SE.	%Specific activity	%Decrease
DHFR: - Control	179.92 ± 1.5	100	0
-50 µg/ml	132.72 ± 1.3	73.77	26.23
TP: - Control	19.63 ± 0.39	100	0
-50 µg/ml	11.27 ± 0.61	57.41	42.59

Table 6: Effect of IC_{50} (50 µg/ml) of *Peganum harmala* alkaloids on the amount of carbohydrate content (µg/ml) at log-phase (96 hrs).

Treatment	Glucose content µg/ml Mean* ± SE.	%Glucose	%Residue
Control	97.10 ± 3.8	100	0
50 µg/ml of the alkaloid	114.52 ± 2.2	117.94	17.94

Table 7: effects of IC_{50} (50 µg/ml) of *Peganum harmala* alkaloids on the specific activity of *Leishmania* FRD-NADH and SDH at log-phase (96 hrs).

Enzyme		Specific activity** Mean* ± SE.	%Specific activity	%Decrease
FRD:	Control	328.40 ± 0.88	100	0
	50 µg/ml	320.36 ± 1.1	97.55	2.45
SDH:	Control	284.05 ± 4.0	100	0
	50 µg/ml	244.43 ± 1.5	86.05	13.95

3- Determination of LD_{50} in Balb/c mice

In vitro antilieshmanial activity of *Peganum harmala* alkaloids was encouraging and prompted us to confirm the toxicity *in vivo*, in mice models. Quintal dose-response used extensively to evaluate toxicity of new chemicals or compounds *in vivo* (Doull *et al*., 1986). In the present research, oral LD_{50} was determined as a first attempt to evaluate toxicity of the alkaloids applying the up-and-down method (Dixon, 1980). According to toxicity rating chart (Depass, 1989) the tested alkaloids were considered to be slightly toxic for mice (1070 mg/kg body wight) (Table 8). Survived mice at sublethal doses (500 and 1000 mg/kg body weight) showed lethargy and prostration, lose of appetite, trembling, walking difficulties and some times, loss of consciousness before recovery or died.

Table 8: Estimated oral LD_{50} of the extracted alkaloids in Balb/c mice.

Measurements	Values
Median lethal dose (mg / kg) body weight	1070
Upper and lower doses (mg / kg) body weight	500 - 3000
Number of mice used	7
Result after 24 hrs.	OOO OXXO
Final dose (mg / kg)body weight	1500

X: death **O:** survival

While this plant has traditionally been used in Bedouin medicine as an emmenagogue and as abortifacient agent (Casey, 1960), there are few reports on its human toxic effects and syndrome (Salah *et al*., 1986).All parts of *Peganum harmala* plant are thought to be toxic. Domestic animals are susceptible to poisoning from this plant, and camels are the most often affected (El-Bahri and Chemli, 1991). Bellil (1983) referred to the digestive and nervous syndromes in animals that consume a sub-lethal amount of the plant. He also illustrated that these animals initially become prostrate and then anorexia, hypersalivation, vomiting and diarrhea occur; in addition to excitability that was followed by trembling, stiffness and accelerated breathing. In Merck Index, Intravenous lethal dose fifty (LD_{50}-iv)

of harmine alkaloid that is isolated from *Peganum harmala* seeds is 38mg/kg in mice, while subcutaneous lethal dose fifty (LD_{50}-sc) is 200mg/kg in rats (Budavari and O`Neil, 1996).

On the other hand, Puzii and Serov (1983) revealed that the extract of *Peganum harmala* is a natural drug, which does not infiltrate in muscles of cattle. Mirzaei (2007) pointed out the therapeutic effects of *P. harmala* extract for the treatment of tropical theileriosis in cattle. i.e. *P. harmala* extract has a significant antitheilerial activity on *Thyleria annulata* (protozoan parasite) *in vivo*. Lala *et al*., (2004) demonstrated that the alkaloid harmine found in *Peganum harmala*, proved to have appreciable efficacy in destroying the intracellular parasite (lieshmania amstigotes) as well as non-hepatoxic and non-nephrotoxic nature for human. They concluded that harmine, in the vesicular forms, may be considered for clinical application in humans.

Present study concludes that *Peganum harmala* alkaloids show a promising *in vitro* antilieshmanial activity with slight toxicity *in vivo* and can be considered as new lead structures in the search for novel antilieshmanial drug. Therefore, there is a need for further investigation about the efficacy of *Peganum harmala* alkaloids as antilieshmaneal agents *in vivo* (in animals) to determine therapeutic indices of such compounds.

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REFERENCES

AL-Chalabi, K. A. (1986). Effect of paramomycin sulfate on growth and thymine nucleotide metabolism in *Leishmania tropica* promastigotes. Proc. of the 4th Sceint. Conf., *Sci. Res. Counc., Baghdad*, Oct. 23-28, **5**(1), 230-236.

AL-Chalabi, K. A. ; AL-Khayat, B. H. (1986). Properties of dihydrofolate reductase from *Leishmania donovani* and *Leishmania tropica* promastigotes. JBSR., **17**(2), 107-117.

AL-Fahhady, BK. (2006). Effect of some medical herbs on growth and metabolism of different clinical parasitic groups in Iraq. Ph.D. Thesis, College of Education, Tikrit University, Iraq.

AL-Hasany, A. M. (2004). Study the electrochemical behaviour of coenzyme flavine adinine dinucleotide (FAD) and estimation the activity of succinate dehydrogenase enzyme (SDH). M.Sc. Thesis, Collage of Sciences, Mosul University, Iraq.

AL-Healy, H. L. (2000). Effect of chloropromazine on growth and metabolism on promastigote forms of *Leishmania* parasite. M.Sc. Tesis, College of Science, Mosul University.

AL-Janker, M. H. (1999). The molecular effect of cadmium, copper and zinc on the growth and metabolism of free living *Tetrahymena pyriformis* (Ehrenberg, 1830). Ph.D. Thesis, College of Science, Mosul University, Iraq.

AL-Jeboori, T. I. ; Evans, D. A. (1980). *Leishmania spp*. In Iraq. Electrophoretic isoenzyme pattern II. Cuteneous leishmaniasis. *Trans. Roy. Soc.Trop. Med. Hyg*., **74**, 178-184.

AL-Jebouri, N.S. (2002). Study of the effect of purine analoges on growth and metabolism of *Leishmania major* promastigotes. Msc. Thesis, College of Education, University of Tikrit, Iraq.

AL-Jubouri, S. A. (2005). Effect of aqueous extracts of *Nerium oleander* and *Metia azedarach* on growth and metabolism of Leishmania *tropica* promastigotes in vitro. M.Sc. Thesis, College of Science, Mosul University, Iraq.

AL-Khan, H. J. (2001). Effect of aqueous extracts of *Capparis spinosa* and *Citrullus colocynthis* plants on growth and metabolism of *Leishmania major* promastigotes in vitro. Ph.D. Thesis, College of Science, Mosul University, Iraq.

Al-Khayat, B.H. (1981). Studies on some aspects of pyrimidine cnucleotide metabolism of Iraqi species of *Leishmania*. M. Sc. Thesis, College of Science, University of Mosul, Iraq.

AL-Rawi, K. ; Kalaf Allah, A. M. (1980). The Completely Randomized Design, in: "Design and Analysis of Agricultural experiments". House of Books for printing and publication, Mosul, pp.37-94.

AL-Shahaat, N. (1986). "Plants and Medicinal Herbs". Dar AL-Bahaar, Beirut, pp. 140-146.

Baccanari, D.; Phillps, A. ; Smith, S.; Sinki, D. ; Burchalls, J. (1975). Purification and properties of E. coli dihydrofolate reductase. *Biochemistry*., **14**, 5267-5277.

Bellil, H. (1983). Les intoxication d vegetate Chez le dromadaire dans le sud tunisien. These Doct. Vet., 1, 66-72.

Benjamin, C. L. ; German, G. R. (1993). "Students Study Guide Microbiology". Concepts and application. McGrow-Hill. Book Company. pp. 254-248.

Berman, J. D.; Edwards, M.K. ; Grogl, M. (1989). Biochemistry of pentostam resistant *Leishmania*. *Am. J. Trop. Med. Hyg*., **40**, 159-164.

Berman, J.D. (1997). Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin. Infect. Dis*., **24**, 684-703.

Bryant, C. ; Behm, C. (1989). "Biochemical Adaptation in Parasites", Energy metabolism, Chapman and Hall, London, UK. pp.25-69.

Budavari, S. ; O`Neil, MJ. (1996). The Merck Index. 12th edn. CRC press, *Peganum harmala*: 4644-4645.

Burton, K. (1956). A study of conditions and mechanisms of the diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid. *Biochem. J*., **63**, 315-323.

Casey, RC. (1960). Alleged antifertility plants of India. *Indian J. Med Sci*, **14**, 590-600.

Champ, PC.; Harvey, RA. ; Ferrier, DR. (2005). "Lippincotts Illustrated Reviews: Biochemestry". 3rd edn. Lippincot Williams and Wilkins, USA. pp. 378-381.

Chan-Bacab, M. J. ; Pena-Rodriguiz, L. M. (2001). Plant natural products with Leishmanicidal activity. *Nat. Prod. Rep*., **18**, 674-688.

Chang, KP.; Fong, D. ; Bray, RS. (1985). "Biology of *Leishmania* and Leishmaniasis" Elsevier Science Publishers, B.V. (Biomedical division). USA. pp.166-173.

Chen, M. ; Zhai, L.; Christensen, S. B.; Theander, T. G. ; Kharazmi, A. (2001). Inhibition of fumarate reductase in *Leihmania major* and *Leishmania donovani* by chalcons. *Antimicrob. Agents. Chemother*., **45**(7), 2023-2029.

Croft, S. L. (2001). Monitoring of drug resistance in leishmaniasis. Tropical Medicine and International Health., 6 (II), 899-905.

Daneil, W. W. (1983). "Biostatistics. A Foundation for Analysis in the Helth Science". 3rd edn. John Wiley and Sonns. New Yourk. pp. 87-89.

Denicola-Seoane, A.; Rubbo, H. ; Prodanov, E. ; Turrens, JF. (1992). Succinate-depending metabolism in *Trypanosoma cruzi* epimastigotes. *Mol. Biochem. Parasitol*., **54**, 43-50.

Depass, L.R.(1989). Alternative approaches in median lethality (LD50) and acute toxicity testing. *Toxicol. Lett*., **49**, 159-170.

Dixon, W.J. (1980). Efficient analysis of experimental observations. *Ann. Rev. Toxicol*. , **20**, 441-462.

Doull, J.; Klassen, C. D. ; Amdur, M. O. (1986). "Casarett and Doull`s Toxicology". The basic science of poisons. 3rd ed. Macmillan publishing Company. Inc. Toronto. pp.25-38.

Eibl, H. (2000). "Cure for Fatal Tropical Disease-Oral Treatment of Leishmaniasis". Max Planck Institute for Biophysical Chemistry, UK.

EL-Bahri, L ; Chemli, R. (1991). *Peganum harmala* L: Poisonous plant of North Africa. *Vet. Hum. Toxicol*. **33**, 276-277.

Evans, WC. (1997). "Treaus and Evans Pharmacognosy". 4th edn. W. B. Saunders Company limited, V. P. India: pp.340-408.

Fernando, HZ. ; Dolores, GP. (2001).*Tryponosomal* dUTPases as potential targets for drug design. Current protein and peptide scence., 2, 389-397.

Hassan, H.F. (1979). Studeis on thymidine nucleotide metabolism in *Leishmania*. MSc. Thesis, College of Science, Mosul Unive Iraq.

Hellemond, V.J. ; Tielens, AG. (1997). *Leishmania* promastigotes have a poor capacity for anaerobic functioning, depend on respiration for their energy generation and inhibition of respiration .-induces a metabolia arrest that enables them to survive anoxic conditions First world congress on leishmaniasis abstracts. *Acat Parasitologica Turcica*. **21**(1), 142.

Herbert, D.; Philips, P. J. ; Stance, R. E. (1971). "In Methods in Microbiology". (5B). (Ed. Norris) J.R. and Ribbons D. W. Academic Press. London and New York. pp.241-245.

Iverson, TM.; Luna, CC.; Chccini, G. ; Ress, DC. (1999). Structure of *Escherichia coli* fumarate reductase respiratory complex.. *Science*., **284**, 1961-1966.

Kamel, S.; Ibrahim, L.; Afifi, A. ; Hamza, S. (1970). Alkaloidal constituents of the Egyptian plant. *Peganum Harmala*. UARJ, Vet Sci., **7**, 71-86.

Kharmazmi, A.; Chen, M.; Zhia, L. Nielsen, SF. ; Christensen, SH. (1997). Discovery and development of oxygenated chalcones A5 potential novel antileishmanial agents. First world congress on leishmaniasis abstracts. *Acat Parasitologica Turcica*. **21** (1), 371.

Lala, S.; Pramanick, S.; Mukhopadhyay, S.; Bandyopadhyay, S. ; Basu, MK. (2004). Harmine: evaluation of its antileishmanial properties in various vesicular delivery systems. *Journal of Drug Targeting*. **12** (3), 165-75.

Li Y.; Liang, F.; Jiang, W.; Yu, F.; Cao, R.; Ma, Q.; Dai, X. ; Jiang, J. ; Wang , Y. ; Si, S. (2007). DH334, a beta-carboline Anti-Cancer Drug, Inhibits the CDK Activity of Budding Yeast. *Cancer Biol Ther*. **6**(8), 17-19.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. ; Randall, R. J. (1951). Protein measurement with the Folin-phenol reagent. *J. Biol. Chem*., **193**, 265-275.

Majumdar, U. K.; Gupta,M.; Chowdhury, S. ; Saha, A.K. (1993). Antileishmanial activities of mycotoxine MT 81 and its derivatives. *Indian J. Exp. Biol*., **31**, 888-890.

Mathews, C.K.; Scrimgeour, K. G. ; Huennekens, F. M. (1963). Dihydrofolic acid reductase. Eds,: Colowick, S. P. and Kaplau, N.O. " Methods in enzymology". 1,364-368.

Mirzaei, M. (2007). Treatment of natural tropical theileriosis with the extract of the plant *Peganum harmala*. *Korean J. Parasitol*., **45**(4), 267–271.

Misra, P.; Khaliq, T.; Dixit, A.; Sen Gupta, S.; Samant, M.; Kumar, A.; Kshawaha, PK.; Majumdar, HH.; Saxena, AK.; Narender, t.; Dube, A. (2008). Antileishmanial activity mediated by apoptosis and structure-based target study of peganine hydrochloride dehydrate: An approach for rational drug design. *J. antimichrobial Chemotherapy*. **62**(5), 998-1002.

Pealing, S. L.; Black, A.A.; Manon, F. D.; Ward, F. B.; Chpman, S. K. ; Reid, G. A, (1992). *Biochemistry*., **31**, 12132-12140.

Puzii, A. D. ; Serov, V. M. (1983). Persistence of pegarmin (apreparation of the alkaloids of *Peganum harmala* in the body of cattle). *Veterinaria*. **5**, 62–64.

Salah, NB. ; Amamou, M.; Jerbi, Z.; Salah, FB. ; Yacoub, M. (1986). Un cas de surdosage en *Peganum harmala* L. *J. Toxicol. Clin. Exp*, **6**, 319-322.

Santhamma, KR. ; Bhaduri, A. (1995). Characterization of respiratory chain of *Leishmania* d*onovani* promastigotes. *Mol. Biochem. Parasitol*., **75**, 43-53.

Schneider, W. C. (1957). Determination of nucleic acids in tissues by pentose analysis in: " Methods in Enzymology". Vol. 111, Academic Press. New York. pp.515-517.

Schweitzer, BI.; Dicker, AP. ; Bertino, JR. (1990). Dihdrfolate reductase as a therapeutic target. *The FASEB J*., **4**, 2441-2452.

Sousek, J.; Guedon, D.; Adam, T.; Bochorakova, H.; Taborska, E.; Valka, I. ; Simanek, V. (1999). Alkaloids and organic acid content of eight Fumaria species. *Phytochemical Analysis*, **10**, 6-11.

Tobie, E. J. ; Brand, T. V. ; Mehlman, B. (1950). Cultural and physiological observations on *Trypanosoma rhodesiense* and *Trypanosoma gambiense*. *J. Parasitol*., **36**, 48-54.

WHO (2001). World Health Organization Division of Control of Parasitic Diseases. Geneva, Switzerland.

WHO. (2002). Leishmaniasis. World Health Organization. Geneva, Switzerland.

Wright, CW. ; Phillipson, JD. (1990). Natural products and the development of selective antiprotozoal drugs. *Phytother. Res*.; **4**, 127–139.

Zuccotto, F.; Zvelebil, M.; Lakowski, R.A. Thornton, J.M. ; Gilber, I.H. (1998). Dihydrofolate reductase as a potential target in *Trypanosomes* and *Leishmania*. *J. Comput-Aided Mol. Des*., **12**, 241-257.