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# Effect of Lupeol on Inducing the Nitric Oxide Production in Macrophages Infected with *Leishmania donovani*

**Talib Fadhil Abbas[1] , Hayder Zuhair Ali[2]**
[1]*College of Pharmacy, University of Al-Muthana, Samawa, Iraq*
[2]*Department of Biology, College of Science, University of Baghdad, Al-Jaderiya Campus Block A, Risafa district, Baghdad, 10071, Iraq*

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**Abstract**

Visceral leishmaniasis is a neglected tropical disease on the rise in different regions of Iraq, especially in areas with poor hygiene and among refugee populations. The effectiveness of existing chemotherapy for leishmaniasis is constrained by its high toxicity, cost, and the development of drug resistance. The current research examined various concentrations (ranging from 125 to 1000 μM) of lupeol to evaluate its ability to boost the generation of nitric oxide, which has anti-leishmanial properties, in an ex-vivo macrophage model. Griess assay was used to detect the nitric oxide (NO) production in *Leishmania donovani* infected U937 cell-line macrophages along 24 and 48 hours post treated. The nitric oxide concentration was significantly increased ($P \leq 0.05$) in the treated infected-macrophages after 24 and 48 hours post treated. Furthermore, the infectivity index was calculated for ex vivo amastigote-macrophage infection and the results showed a significant decrease in the percentage of invasion at higher concentrations of Lupeol at all periods of incubation ($P \leq 0.05$); whereas, the average of amastigotes per cell in lupeol-treated macrophages increased significantly ($P \leq 0.05$) only after 48hours of incubation. The results indicate that lupeol has the potential to enhance anti-leishmanial nitric oxide production by macrophages, enabling them to eliminate intracellular amastigote forms of the parasite. Further details on effect of Lupeol can be studied as a promising anti-leishmanial compound.

**Keywords**: visceral leishmaniasis, Lupeol, Nitric oxide, cell line

# تأثير الليوبيول في تحفيز إنتاج أكسيد النيتريك في الخلايا البلعمية المخمجة بطفيلي اللشمانيا الاحشائية

**طالب فاضل عباس[1] , حيدر زهير علي[2]**
[1] كلية الصيدلة, جامعة المثنى, السماوة, العراق.
[2] قسم علوم الحياة, كلية العلوم, جامعة بغداد, الجادرية البناية A,الرصافة, بغداد10071,عراق.

**الخلاصة**

يعد داء الليشمانيات الحشوي من امراض المناطق الاستوائية المهملة والذي يزداد انتشاره في مناطق مختلفة من العراق، لاسيما في المجتمعات التي تعاني من قلة العناية الطبية، وفي مناطق اللاجئين . ان الفعالية العلاجية للعلاج الكيميائي الحالي لداء الليشمانيات محدودة بسبب السمية العالية والكلفة وظهور مقاومة الأدوية .

*Email: taleb.fadel2102@sc.uobaghdad.edu.iq

في الدراسة الحالية، تم استخدام تراكيز مختلفة (125− 1000) مايكرومولاري من عقار الليوبيول لتحديد قدرته على تعزيز إنتاج أكسيد النيتريك المضاد لليشمانيات في نموذج خارج الجسم الحي في خط خلايا البلاعم العملاقة .وقد استخدم اختبار كريس للكشف عن إنتاج أكسيد النيتريك (NO) في الخط الخلوي للبلاعم العملاقة U937المصابة بطفيلي *Leishmania donovani* على مدار 24 و48 ساعة بعد المعاملة. اثبتت النتائج زيادة في تركيز أكسيد النيتريك بشكل ملحوظ في البلاعم العملاقة بعد المعاملة .فضلا عن ذلك، تم حساب مؤشر الخمج لعدوى البلاعم العملاقة خارج الجسم الحي وأظهرت النتائج انخفاضا معنويا ($P\leq0.05$)في نسبة الاصابة في جميع التراكيز ، ومتوسط عدد الاطوار عديمة السوط في الخلية الواحدة في البلاعم العملاقة المعاملة بالمركب فقط بعد 48 ساعة من فترة التعرض. تشير هذه النتائج إلى أن الليوبيول لديه القدرة على تحسين إنتاج أكسيد النيتريك المضاد لليشمانيات بواسطة البلاعم العملاقة ،مما يسمح له بقتل الاطوار عديمة السوط داخل الخلايا . يمكن دراسة المزيد من التفاصيل حول تأثير الليوبيول باعتباره مركبًا واعدًا مضادًا لطفيلي اللشمانيا.

## 1.Introduction

Leishmaniasis is a group of parasitic diseases classified as 'Neglected Tropical Diseases' [1]. Over twenty identified species of the Leishmania genus can cause infection in humans [2]. Iraq is considered endemic with cutaneous and visceral leishmaniasis as part of middle east epidemiology [3, 4]. During the initial stages of an infection, macrophages produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) as a defense mechanism to eliminate invading pathogens while leaving the host cell unharmed [5]. This enzyme-rich process may be induced by phagocytosis. NADPH oxidase 2 (NOX2) and inducible nitric oxide synthase (iNOS) generate superoxide (O2–) and nitric oxide (NO) in macrophages [6]. *Leishmania* parasites use the lipophosphoglycan (LPG) shield to avoid ROS and RNS and delay NOX2 assembly at the phago-lysosome surface, halting the O2 generation [7]. Intracellular amastigotes can stimulate macrophages to manufacture arginase, which competes with iNOS for arginine and creates necessary nutrients for the parasites, such as l-ornithine for polyamine and urea synthesis, while lowering parasite-toxic NO [8]. Only a few drugs like allopurinol, amphotericin B formulations, ketoconazole, paromomyxin, miltefosine and pentavalent antimonials (Pentostam) have been tested in clinical trials since their introduction. Since leishmaniasis treatments are limited, new compounds are needed to reduce morbidity and mortality of infected patients treated with the available drugs [9]. Previous research has demonstrated that traditional medicinal treatments used in endemic regions can contain new classes of bioactive compounds with anti-infective potential [10, 11]. One such compound is the pentacyclic triterpene lupeol, which is found naturally occurring in numerous plant species [12]. The pharmacological molecules of *Emblica Officinalis*, *Bombax ceiba*, *Walsura trifolita Leucaena leucocephala* plant effects can target microbial infections as lupeol, including intracellular eukaryotes, arthritis, renal issues, diabetes, hepatotoxicity, cardiovascular disease, and cancer [13].

Treatment varies on *Leishmania* species, clinical appearance, and patient features [14]. Current leishmaniasis therapy encounters resistance, limited access to effective treatments, and the need for better patient alternatives. To fill these gaps, researchers are investigating new compounds and improving existing treatments [15]. Testing drugs for leishmaniasis effectiveness and safety begins in vitro[16]. Medication effectiveness in treating leishmaniasis may vary due to treatment sensitivity of different *Leishmania* species, patient immunological state, or drug pharmacokinetics [17]. Nitric oxide (NO) generation is a common immunomodulatory measure for parasite clearance as NO is the main killer of *Leishmania* invaders [18]. It may be detected directly by nitrite content in the culture supernatant or indirectly by iNOS gene expression [19].

Earlier research has demonstrated lupeol's efficacy in eliminating malaria, trypanosomiasis, and leishmaniasis protozoa by reducing parasite load and eliciting a robust immune response [20]. Previous studies referred that lupeol from Perabenensis inhibited various *Leishmania* species, according to Fournet *et al.* [21] and Sohag *et al.* [22]. This compound exhibits an $IC_{50}$ value of 100µg/mL against five *Leishmania* species, including cutaneous or visceral for both promastigotes and amastigotes [23, 24]. One potential alternative to the current treatment protocols for leishmaniasis is lupeol, a chemical having comparable antileishmanial effects to Amphotericin-B with lower toxicity [25]. NO production is important for *Leishmania* eradication inside host-cell macrophages. Many metabolites can modulate macrophage activities, including NO release, with varying effects depending on experimental conditions [26]. This study aimed to assess lupeol's ability to indirectly inhibit the parasite by examining its effects on nitric oxide (NO) production in an ex vivo model of *L. donovani*-infected macrophages. Specifically, it investigated whether lupeol could enhance NO secretion by infected cells as a potential anti-parasitic mechanism of action.

## 2.Materials and Methods:

### *2.1. Leishmania isolate and U937 cell-line*

This study utilized an Old-World Iraqi strain of Leishmania donovani, with the isolate number (MHOM/IQ/2005/MRU15), which was obtained from the parasitology laboratory for postgraduate studies at the Department of Biology, College of Science, University of Baghdad [27]. For ex vivo infection, the promonocytic human myeloid leukaemia cell line (U937) was used, it was purchased from Rawafid-Aleloom company for research services, Hillah-Iraq.

### *2.2. Lupeol preparation*

Lupeol powder was obtained from Sigma-Aldrich (Purity ≥94%) and prepared as per the manufacturer's instructions by dissolving 2 mg in 1000 µl of Dimethyl sulfoxide (DMSO-Sigma-Aldrich). A stock solution of 5000µM was prepared for later experiments.

### *2.3. Parasite culture*

Promastigote forms of *Leishmania donovani*was cultured in RPMI 1640 liquid media (Gibco, UK) in cell culture flasks with a filter cap of 25 $cm^2$ (Falcon/USA) at a pH of 7.4, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, UK). In addition, 100 IU/mL of penicillin and 100 mg/mL of streptomycin (Gibco, UK) was added to media. The culture maintained at a temperature of 26ºC[28]. The cultures were regularly subcultures and observed under a light microscope [27] to monitor their growth and viability.

### *2.4.U937 cell line Culture*

The U937 cell line was cultured in RPMI1640 (Gibco, UK) media supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) filtered using a double sterile syringe filter with pore sizes of 0.22 micron supplemented with 1% penicillin/streptomycin, HEPES buffer 1%. The cells were incubated at 37°C in a humidified incubator with 5% (CO2)[27].

### *2.5. Nitric oxide (NO) measurement by Griess Reagent System assay*

Nitrite accumulation in the culture of infected U937 macrophages was detected using the Griess assay according to the kit and previously reported [29]. Briefly, U937 macrophages ($1x10^6$ cell/ml) were infected with *L. donovani* promastigotes at a ratio of 1:10. The infected macrophages were subsequently treated with different concentrations of lupeol (1000, 750, 500, 250, 125) µM for 24 and 48hours. For each concentration, the experiment was conducted in triplicates. Cell-free supernatants were collected from two sets of infected macrophages: one set that received no treatment and another set treated with lupeol. A portion of 150 µl of the liquid remaining after centrifugation was combined with 130 µl of deionized water and 20 µl

of Griess reagent (Sigma-Aldrich). The mixture was then allowed to incubate for a duration of 30 minutes. The absorbance at 490 nm was measured using the Biotek micro-platereader. The standard curve for nitrite was established by dissolving sodium nitrites in distilled water at values ranging from 10 to 300 Micromolary (µMol). The findings were measured in micromoles of nitrite.

*2.6 .Giemsa staining and infectivity calculation*

The effect of lupeol on intracellular amastigotes can be studied by *L. donovani* promastigotes infected the host macrophages (the U937 cell line); in ratio of macrophages to parasites 1:10. In a humidified incubator with 5% CO2, the infected cells were then grown at 37ºC. The infected macrophages were subjected to several doses (125, 250, 500, 750, and 1000) µM of lupeol in triplicate on a 24-well flat surface plate (SPL/Korea) after being incubated for 2 hours in a humidified incubator, while a control group was left untreated. After 24 and 48 hours, the cultures were removed. The efficacy of lupeol was evaluated by calculating the infectivity index by counting the percentage of infected cells, the percentage of amastigotes in 100 macrophages and the average of parasite per cell [30]. There was a comparison between the intracellular load of parasites in treated and untreated macrophages. The samples in the 24-well plate were first stabilized by immersion in 100% methanol for 2 minutes. After drying naturally, the samples were stained with Giemsa for 3 minutes. They were then rinsed with phosphate-buffered saline (PBS) to remove excess stain. At a magnification of 40x, the colored plate wells were analyzed by inverted microscopy. In order to determine the number of macrophages in the field, at least 100 were counted in each well for infectivity index calculation [28].

*2.7. Statistical analysis*

Prism graph unpaired t test was used for comparison between treated and untreated parasites, significant at p value $\leq 0.05$.

## 3.Results

*3.1.Lupeol affects nitric oxide production by macrophages infected with Leishmania donovani.*

The Griess assay analysis revealed a notable increase in NO production by host cells in lupeol-infected macrophages compared to non-treated infected macrophages across all concentrations studied. The maximum NO concentration was at 500 µM after 24 hours and at 1000 µM after 48 hours, 188 and 435 µMol, respectively, (Figures 1 and 2).

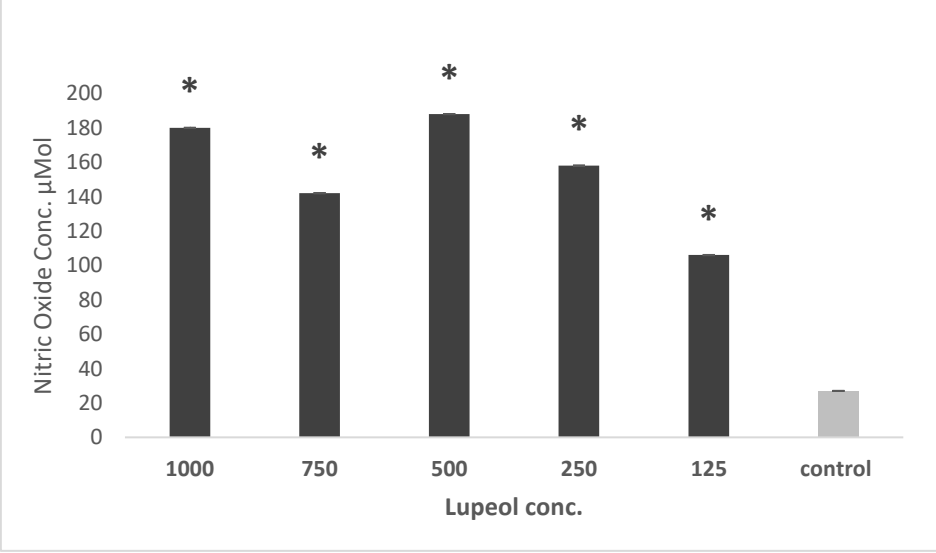



**Figure 1:** Nitric oxide production in U937 culture infected by *L. donovani* and treated with Lupeol at 24 hours incubation time, (*) = p value $\leq 0.05$.

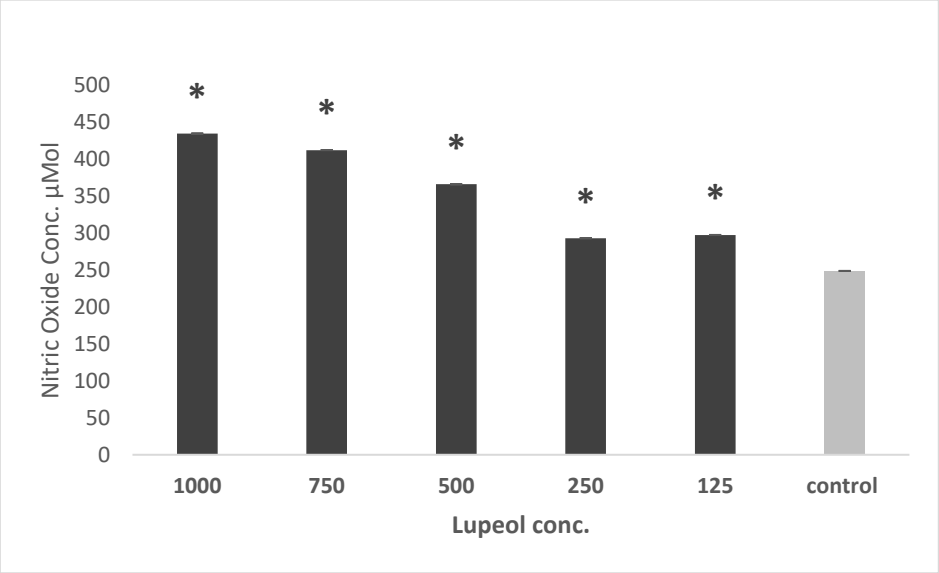



**Figure 2:** Nitric oxide production in U937 culture infected by *L. donovani* and treated with Lupeol at 48hours incubation time, (*) = p value ≤ 0.05.

*3.2. Infectivity index of Giemsa staining*

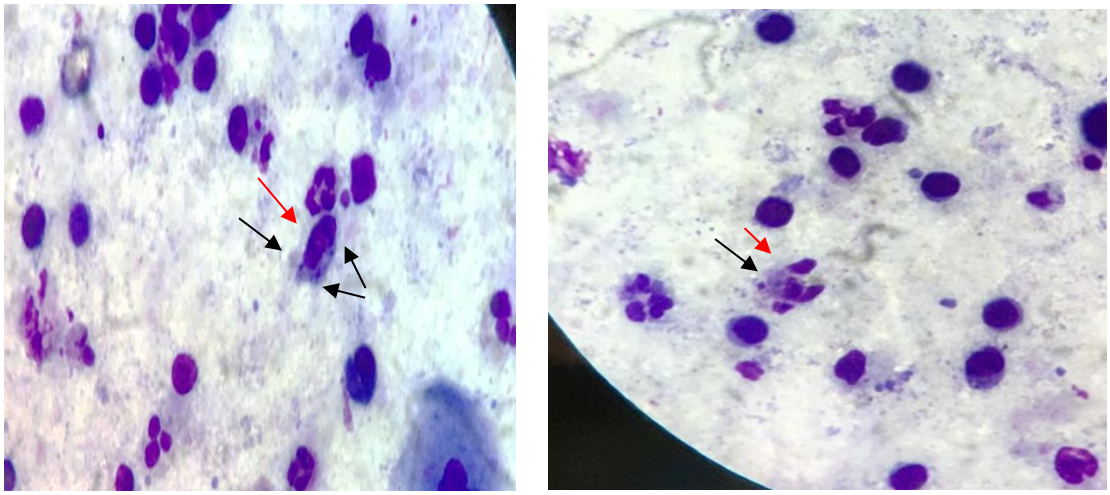
The infectivity index revealed the inhibitory efficiency of lupeol in reducing the invasion of infectious parasites into macrophages in cells treated with lupeol, when compared to untreated cells. There was a significant difference (P≤0.05) the proportion of infected macrophages and percentage number of amastigotes in 100 macrophages at doses ranging from 1000-250 µM after 24 hours and 1000-500 µM after 48 hours. Furthermore, the quantification of the percentage of invasive amastigote per single macrophage was minimized in treated cells less than the controls at all studied concentrations (Table 1 and 2). Giemsa staining of treated and non-treated macrophages are shown in (Figure 3).

**Table 1:** Infectivity index of U937 infected with *L. donovani* treated or untreated with lupeol for 24 hours, (*) = p value ≤ 0.05.

| Parameter | 1000µM | 750 µM | 500µM | 250 µM | 125µM | control |
|---|---|---|---|---|---|---|
| % of infected macrophages | 30% * | 42% * | 67% * | 76% * | 84% | 90% |
| % of amastigote /100 Macrophages | 35% * | 45% * | 75% * | 80% | 90% | 110% |
| % of amastigote /cell | 1.17 | 1.07 | 1.12 | 1.05 | 1.07 | 1.3 |

**Table 2:** Infectivity index of U937 infected with *L. donovani* treated or untreated with lupeol for 48 hours, (*) = p value ≤ 0.05.

| Parameter | 1000µM | 750 µM | 500µM | 250 µM | 125µM | control |
|---|---|---|---|---|---|---|
| % of infected macrophages | 35% * | 60% * | 80% * | 85% | 90% | 95% |
| % of amastigote /100 Macrophages | 41% * | 70% * | 83% * | 89% | 95% | 140% |
| % of amastigote /cell | 1.17 | 1.17 | 1.04 | 1.05 | 1.06 | 1.47 |



(A) (B)

**Figure 3**: U937 macrophage cell line infected with L. donovani, (A) non-treated cells, (B)Lupeol-treated cells. Red arrow = infected macrophage, black arrow = intracellular amastigotes, Giemsa stain, 1000X.

**4.Discussion:**

Since nitric oxide (NO) plays a crucial role in leishmaniasis, accurately quantifying its levels would yield valuable insights for further research. However, directly measuring NO was historically challenging due to its short-lived nature and low concentration levels [30]. In the current research, the results of the Griess assay on the old-world *Leishmania donovani* proved the activity of lupeol to improve the production of anti-microbial nitric oxide that is produced by the host cell (macrophage) against the intracellular amastigotes. Data showed that the concentration of NO was the highest after 24 hours post treatment in which it was raised by about 7 folds ($p$ value $\leq 0.001$) at 500 µM. This raising was continued after 48 hours post treatment; however, the NO concentration was about 1.5 folds than that of control.

In previous studies, both the promastigote and amastigote stages of *Leishmania* were inhibited by lupeol. Lupeol-mediated leishmanicidal activity was carried out through breakdown of the cytoplasmic membrane of the *Leishmania donovani*, as revealed by DISC3-mediated fluorometric examination. Additionally, it decreases parasite loads in the liver and spleen and stimulates NO generation in macrophages infected with *Leishmania donovani* [30]. A similar in vitro study investigated the cytotoxic effects of lupeol and found that it exhibited toxic effects against Leishmania promastigotes with an IC50 value of 65 µg/ml [31].

These findings are in line with previous in vitro experiments of Das *et al*. [32] where visceral leishmaniasis showed immunomodulatory and anti-leishmanial properties of triterpenoid lupeol extracted from *Sterculia villosa*. Similar research on a mouse model studied the synergetic effect of both AmpB and lupeol and found that anti-leishmanial and immunomodulatory actions in BALB/c mice infected with *Leishmania donovani* where notablereducing the hepatic and splenic parasite loadconcluding theboosting of nitric oxide, and stimulating Th1 cytokines (IL-12 and IFN-γ) [33].

Another immunological study by Panaro *et al.* [25] found a correlation between the ability of lupeol to kill the invading *Leishmania* with in living organisms viaover-expression of IFN-γ, as lupeol may stimulate Th1 immune response, in addition to its direct action on the parasite.

The researchers observed that infected macrophages treated with lupeol generated elevated levels of $H_2O_2$, which can trigger programmed cell death in intracellular parasites [34].

Another recent study on the flagellated *Trypanosoma cruzi* showed that pentacyclic triterpenes, including lupeol derivatives, were found to compete for enzymatic site of cysteine synthase and other building blocks enzymes suggesting their antagonist activity by inhibiting the key molecules of parasite's defense against oxidative stress [35]. In another study by Santos-Pereira *et al.* [36] of mouse model determined that macrophages increase parasite burden of *Leishmania amazonensis* and was more susceptible to infection by *Leishmania major* when NO production was decreased.

Lupeol effectively stimulated infected host macrophages, inducing a leishmanicidal state. This action, initiated by lupeol, can serve as an additional method to eradicate parasites [38]. Past studies have shown that all human Leishmania species can evade the host's immune defenses and persist within cells by inducing immunosuppression and altering chemokine patterns [37]. As evident from the Results tables, the infectivity index, which measures the degree of infection, was lower in the macrophages treated with the compound compared to the untreated control macrophages. Additionally, all lupeol concentrations reduced the number of internalized amastigotes. Such experimental model evaluates the effects against the parasite by activating microbicidal mechanisms in the host macrophages [38,39]. With a significant decrease in infected macrophages and amastigotes per macrophage, data demonstrated that all treatments achieved successful reduction of intracellular amastigotes. Consequently, these findings offer credibility to inquiries exploring the possible participation of macrophage-triggered pathways in this concept.

## Conclusion:

The outcome of this study revealed that lupeol proved its effectivity against the Old-world Iraqi strain of *Leishmania donovani* by increasing the nitric oxide levels within 24 and 48 hours suggesting its potential to induce the release of nitric oxide (NO). Furthermore, diminishing intracellular infective load. These findings are encouraging and suggest that lupeol has the potential to be studied further for visceral leishmaniasis treatment.

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