Annals of Parasitology 2024, 70(2), 00–00
doi: 10.17420/ap7002.523

Original paper

In vitro efficacy of different concentrations of lupeol on old world *Leishmania donovani*

Talib F. ABBAS[1], Hayder Z. ALI[2]

[1]College of Pharmacy, University of Al-Muthana, Samawa, Almuthana, Iraq
[2]Department of Biology, College of Science, University of Baghdad, Al-Jaderiya Campus Block A, Risafa district, Baghdad, 10071, Iraq

Corresponding Author: Hayder Zuhair Ali; e-mail: hayder.zuhair@sc.uobaghdad.edu.iq

ABSTRACT. Leishmaniosis is a tropical neglected parasitic disease that is endemic in many countries, including Middle East, with no existing effective vaccines. The bite of female sand-fly transmits the causative agent, *Leishmania* spp., to humans. High toxicity, resistance and treatment failure of the available chemotherapy against visceral leishmaniosis demands the investigation of new anti-leishmanial compounds. Lupeol is a form of triterpene isolated from several medicinal plants and possesses an antimicrobial property. In this study, cytotoxic effect of lupeol was screened against the mammalian amastigotes form and insect promastigote form of *Leishmania donovani,* following three cycles of incubation at different concentrations by MTT assay. Results revealed the *in vitro* anti-leishmanial effect of lupeol on both forms of the parasite where significant decline in promastigotes and amastigotes growth was observed. This was conducted along three times of follow up (24, 48, 72) hours, in comparison to the classical sodium stibogluconate treatment. Cell viability was calculated and the minimum IC_{50} was detected after 48 hours for amastigotes and 24 hours for promastigotes, 12.125 µM, 102.78 µM, respectively. Given the severity of visceral leishmaniosis and the toxicity of conventional chemotherapies, the anti-leishmanial activity of lupeol suggested a promising compound for additional clinical trials.

Keywords: lupeol, MTT assay, visceral leishmaniosis, Iraq

Introduction

Leishmaniosis is one of neglected parasitic diseases around the world, caused by flagellated protozoan *Leishmania* which is predominantly prevalent as cutaneous or more serious visceral form [1]. More than 70 tropical and sub-tropical countries are generally affected by leishmaniosis where *Phlebotomus* vector is mostly thriving [2]. Moreover, children account for about 50% of reported cases of cutaneous and visceral leishmaniosis (VL) [3]. Clinically, the cutaneous form is most common, whether old or new world, and is rarely becoming life-threatening, however, permanent scars or disfiguring skin ulcers can be developed [4,5]. In Asia and Africa, visceral leishmaniosis or kala-azar is caused by old-world species of *Leishmania donovani* and/or *L. infantum* with an estimation of 500,000 new cases are reported annually [6]. Symptoms of visceral leishmaniosis is critical if untreated, where enlargement of viscera, pancytopenia, high liver enzymes and hypoalbuminemia are progressed before death [7].

Despite the fact that there have been some promising leishmanial-vaccination trials in mouse models, there is currently no approved immunization against human visceral leishmaniosis [8]. In 2021, the World Health Organization called for a new road map of neglected tropical diseases, which involved new therapeutic insights for various parasitic infections, including leishmaniosis [3]. Giving conventional systemic chemotherapies for VL, such as antimony and amphotericin B, may have toxic or cardiovascular side effects and posing potential resistance [9,10]. In recent years, many studies have been conducted to examine novel treatments and diverse compounds that are less

harmful and more readily available than currently utilized chemotherapies [11–13]. Lupeol and its derivatives are kinds of triterpenoids, derived from several edible plants, proved to have anti-inflammatory and antimicrobial activity, including protozoa [14,15]. Previous studies examined the *in vitro* and *in vivo* lupeol effect on *Leishmania* and demonstrated its anti-leishmanial and immune-modulatory properties [16,10,14]. Additionally, lupeol was reported to upregulate T helper-1 cytokines in mouse models, while membrane damage was detected post promastigote treatment. Using lupeol in combination with amphotericin B verified low parasite load in the viscera of infected BALB/c mice [16]. Both cutaneous and visceral leishmaniosis are endemic in Iraq, in which old-world *L. donovani* and *L. infantum* are responsible of reported cases [1,12]. The available chemo-therapy of pentavalent antimony is the only drug available in Iraqi hospitals, where about 50% of VL cases are of children below 10 years old [17].

In the current study, the cytotoxic effect and viability were investigated against *in vitro* promastigote and axenic culture of amastigote after three times follow up of lupeol treatment on of the old-world Iraqi strain of *Leishmania donovani.*

Materials and Methods

Parasite culture

Previously identified *Leishmania donovani* isolate (MHOM/IQ/2005/MRU15) was used at the parasitology laboratory of postgraduates, College of Science, University of Baghdad [12]. Promastigotes culture was grown in RPMI 1640 media (Gibco/UK) supplemented with 10% heated inactivated fetal bovine serum (Sigma/USA), 1% pen/strep (Gibco/UK), at 26°C. Procyclic promastigote was differentiated to axenic amastigotes according to [12] where pH was adjusted to 5.5 and incubated at 35°C.

Chemicals

Lupeol preparation: lupeol (purity ≥ 94%) was purchased from Sigma Aldrich and dissolved in DMSO (2mg/ml) according to the manufacturer's, stock of 5000 µM was prepared for later experiment. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide was purchased from (Sigma/USA). Sodium stiboglocunate (pentostam) 100mg/ml (RMPL Pharma/India).

Anti-promastigote and axenic amastigote cytotoxicity

Cytotoxicity screening of lupeol was conducted against the two forms of the parasite. Log phase of promastigote and amastigote cultures were counted under high power of light microscope in a haemocytometer chamber (Nunc®) then pipetted in 96-well plate (SPL/Koria) in a concentration of 1×10^6 cell in 100 µl/well. Lupeol stock of 2000 µM was prepared in an Eppendorf tube and a serial dilution was followed in the well-plate starting from 1000 µM to end with 15.6 µM. Triplicate wells for each concentration was made for both promastigote and amastigote plates. Promastigote plate was incubated at 26°C while amastigote plate at 35.5°C for 24, 48 and 72 hours before MTT assay examination, DMSO was used as a negative control [12,18]. In parallel plates of promastigotes and amastigote as described above, sodium stibo-gluconate (SBB) was used as a positive control at the same concentrations and serial dilutions as lupeol's.

Diphenyl tetrazolium bromide colorimetric (*MTT*) *method*

According to the manufacturer's and previously published [19], 10 µl of MTT solution was added to each well and incubated for further four hours before 20 µl of DMSO was added as a stop solution to dissolve formazan crystals that were created during the experiment. Results of all plates were read by ELISA reader (Biotek®) at 570 nm. Cell viability was analyzed by dividing absorbency of treated cells on average of untreated cells, for each concentration. IC_{50} were calculated by plotting cell viability against log lupeol concentration [12,20].

Statistical analysis

Prism graph unpaired t test was used for comparison between treated and untreated parasites for both lupeol and sodium stibogluconate, significant at P-value ≤ 0.05.

Results

Cytotoxicity and cell viability of promastigotes

In vitro active proliferating insect form of *Leishmania donovani* growing at 26°C was treated with serial concentrations of lupeol (1000, 500, 250, 125, 62.5, 31.2, 15.6) µM and incubated for 3 times of follow up 24, 48 and 72 hours. This was performed alongside the classical antileishmanial

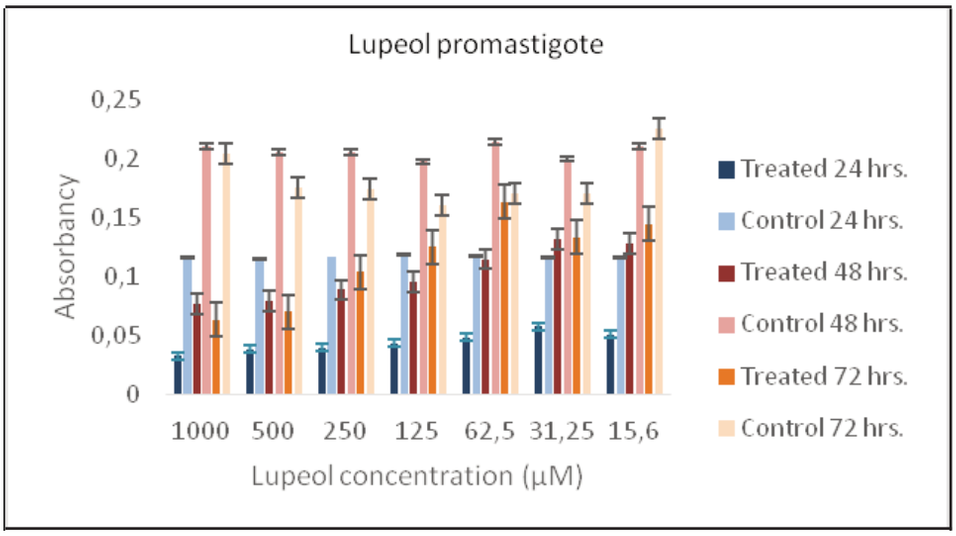

Figure 1. Cytotoxicity of lupeol against promastigotes after 24, 48 and 72 hours

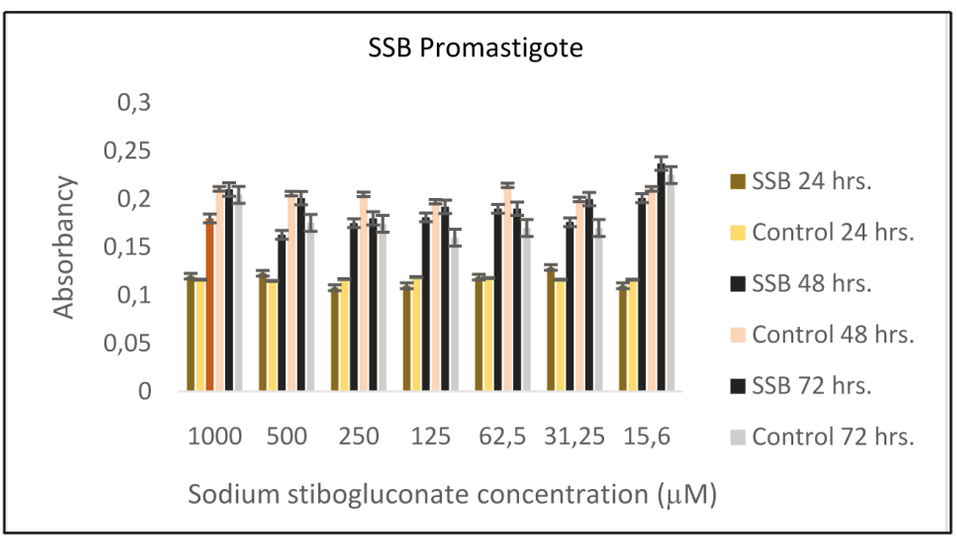

Figure 2. Cytotoxicity of sodium stibogluconate against promastigotes after 24,48 and 72 hours

sodium stibogluconate (SBB), in comparison to the untreated control parasites. The results showed that lupeol inhibited the promastigotes growth at all studied concentrations, with highest effect was noticed after 24 and 48 hours (*P*-value = 0.00001, Fig.1). Counterpart plate of promastigotes treated with sodium stibogluconate showed normal growth with no competitive inhibition at the studied concentrations, where absorbency was very analogous between treated and untreated parasites (Fig. 2).

Cell viability was calculated against logarithm lupeol concentration for the three times of follow up, minimum cell viability was observed after 24 hours treatment for promastigotes, in which growing inhibition less than 50% was seen at all concentrations (Fig. 3).

Analysis of the inhibitory effect of lupeol was detected by calculation of IC_{50} which revealed the lowest after 24 hours, which was 102.78µM. The measured IC_{50} after 48 and 72-hours incubation was (115.88, 284.6) µM, respectively.

Cytotoxicity and cell viability of axenic amastigotes

The axenic mammalian form of *Leishmania donovani* growing at 35.5°C was screened for lupeol activity. The results found that amastigotes proliferation was decreased at all the studied concentrations for the three days of follow up. Following 72 hours incubation revealed the highest anti-amastigote effect (*P*-value = 0.00001, Fig. 4).

Amastigotes treated with sodium stibogluconate was growing normally, with no significance

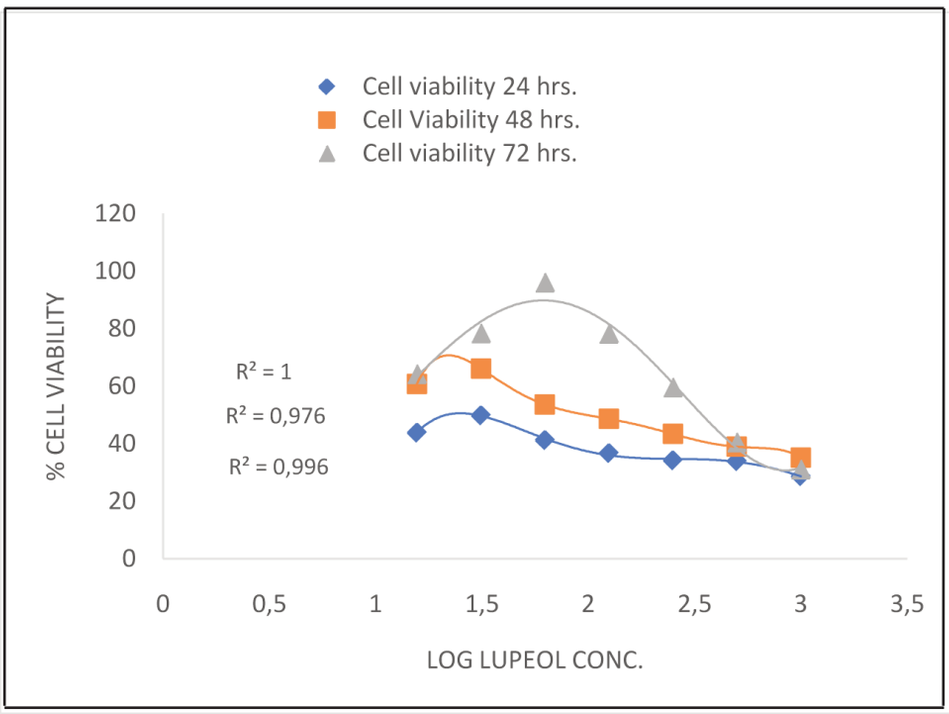

Figure 3. Cell viability of promastigote after lupeol treatment after 24, 48 and 72 hours

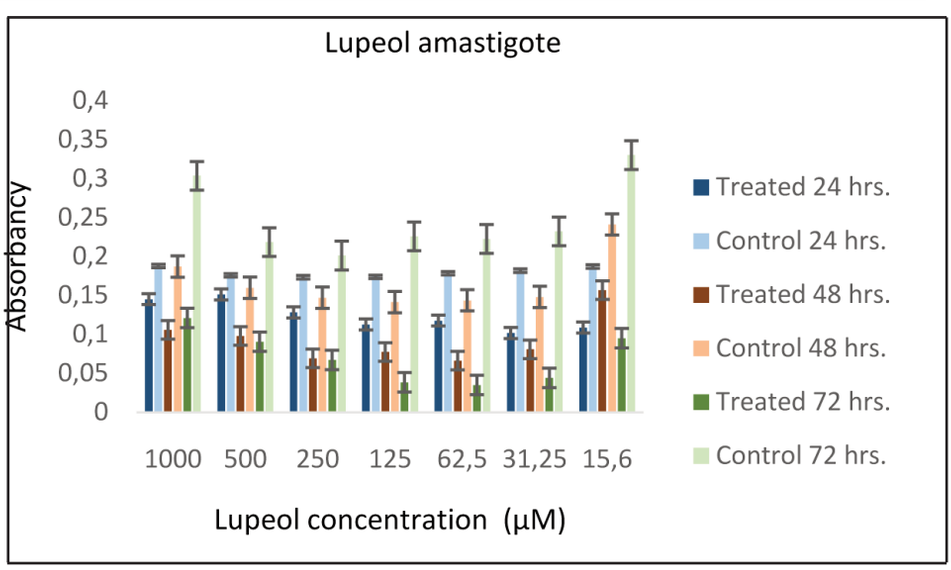

Figure 4. Cytotoxicity of lupeol against amastigotes after 24, 48 and 72 hours

difference in absorbance values between treated and untreated cell was noticed (Fig. 5).

Cell viability of amastigotes was different than that of promastigotes, in which the minimum viability was recorded after 72 hours of treatment (Fig. 6). The inhibitory effect of lupeol against axenic amastigotes was determined by IC_{50} calculation, in which the lowest was 12.125 µM following 48 hours of treatment. IC_{50} was 135.8 µM and 204 µM following 24 and 72 hours of treatment, respectively.

Discussion

Leishmaniosis is one of neglected diseases with growing resistant strains and serious after effects of classical existing therapies including, antimony-based drugs, amphotericin B and paromomycin [21,22]. This study found that highly purified lupeol demonstrated deleterious effect against both promastigotes and axenic amastigotes at all studied concentrations, following six serial dilutions of 1000 µM. Notably, promastigotes showed quick

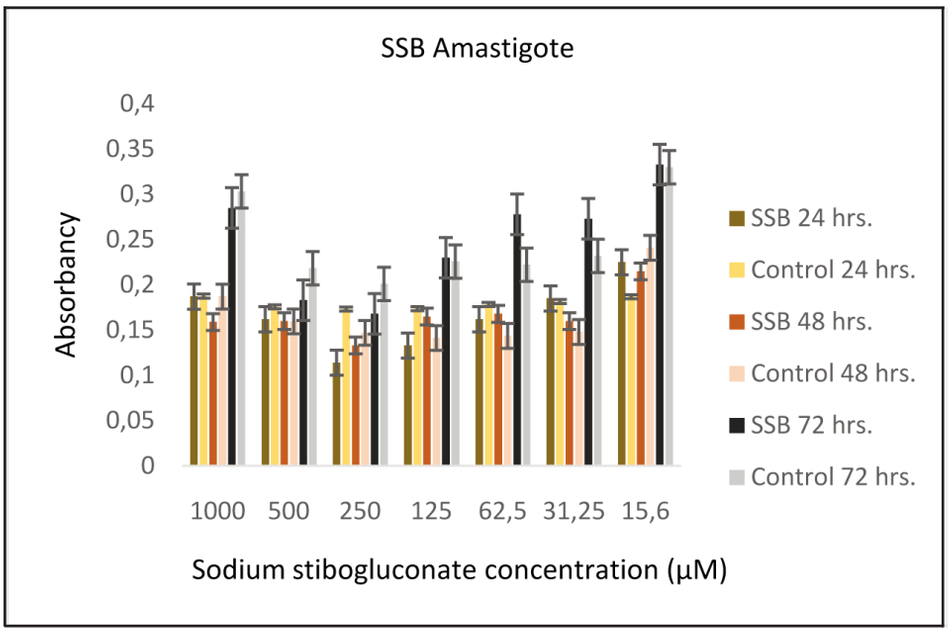

Figure 5. Cytotoxicity of sodium stibogluconate against amastigotes after 24, 48 and 72 hours

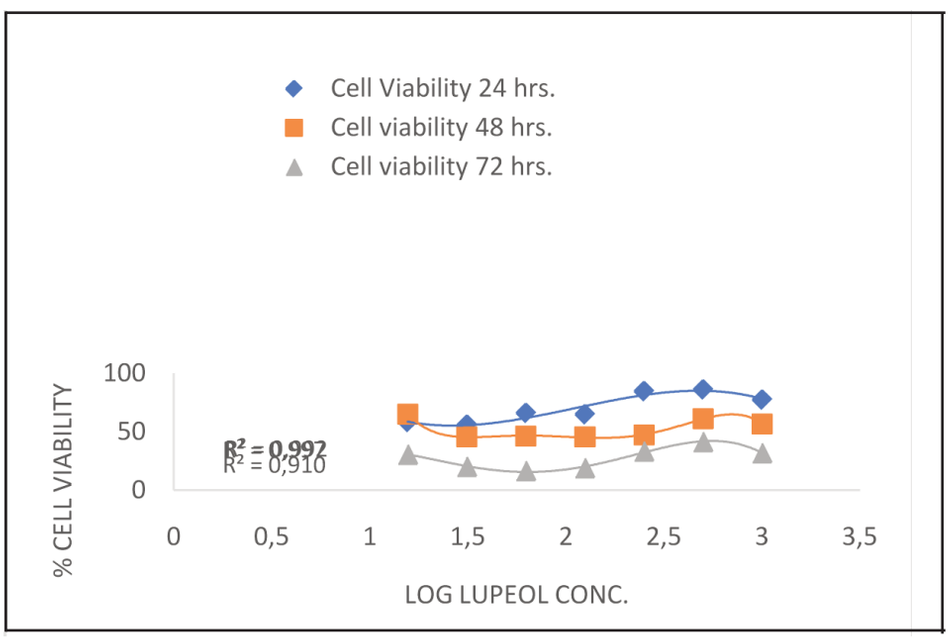

Figure 6. Cell viability of amastigotes after lupeol treatment after 24, 48 and 72 hours

sensitivity to treatment after 24 hours, while the greatest decline of amastigote form was noticed after 72 hours post treatment. Furthermore, the identical plates of promastigote and amastigote, treated with sodium stibogluconate, exhibited normal growth when screened under equivalent conditions and compared to the healthy untreated parasites, along the three days of follow up.

Immunosuppression produced by *L. donovani* to infected people and the toxicity of the available chemicals urge the development of alternative compounds of natural source [10]. Terpenes and terpenoids essential oils are phytochemicals isolated from different plant parts and have powerful antimicrobial characteristics, such compounds can efficiently cross cell membranes [23–25]. Monoterpenes confirmed anti-parasitic properties by altering cellular structure, generating free radicals and triggering apoptosis [26,27].

Lupeol was chosen as it has previously proved its anti-leishmanial activity on the Indian strain of visceral leishmaniosis, however, the *in vitro* assay was only for 2 hours post-promastigote treatment [21]. Additionally, it has been shown to have anti-

trypanosomal activity [28]. In the current work, the IC_{50} calculations displayed the lowest after 48 hours treatment for amastigotes (12.125 µM) while for promastigotes, the lowest was counted after 24 hours (102.78 µM). Interestingly, lupeol was proven to be safe for mammalian cell lines at concentrations less than 234.3 µM [29]. Furthermore, *in vivo* study demonstrated that experimentally infected mice with *L. infantum* exhibited normal biochemical liver function following lupeol treatment [10]. A parallel investigation of lupeol against *L. donovani* in a mouse model, proved the recovery of protective immunity in the infected animals by enhancing delayed type of hypersensitivity (DTH) after lupeol treatment, when compared to classical amphotericin B [30]. Moreover, additional administration of lupeol resulted in elevation of reactive oxygen species generation and nitric oxide within macrophages, in addition, downregulation of cytokines involved in immunosuppression was detected [21,31,32].

This is one of the very first anti-leishmanial investigation of lupeol against the Iraqi strain of old-world *L. donovani,* where its toxic effect against the two forms of the parasite was proved. However, further investigation of *in vivo* study is needed.

References

[1] Majeed B., Sobel J., Nawar A., Badri S., Muslim H. 2013. The persisting burden of visceral leishmaniasis in Iraq: data of the National Surveillance System, 1990–2009. *Epidemiology and Infection* 141(2): 443–446. doi:10.1017/S0950268812000556

[2] Cheghabaleki Z.Z., Yarahmadi D., Karampour M., Shamsipour A. 2019. Spatial dynamics of a Phlebotomine sand flies population in response to climatic conditions in Bushehr Province of Iran. *Annals of Global Health* 85(1): 60. doi:10.5334/aogh.30

[3] Ending the neglect to attain the sustainable development goals: a road map for neglected tropical diseases 2021–2030. WHO. 28 January 2021. https://www.who.int/publications/i/item/9789240010352

[4] Knight C.A., Harris D.R., Alshammari S.O., Gugssa A., Young T., Lee C.M. 2023. Leishmaniasis: recent epidemiological studies in the Middle East. *Frontiers in Microbiology* 13: 1052478. doi:10.3389/fmicb.2022.1052478

[5] Bayram D.M., Hassan G.M., Ali H.Z. 2022. Detection of some CC chemokine ligands in patients with cutaneous leishmaniasis. *Iraqi Journal of Science* 63(4): 1431–1437. doi:10.24996/ijs.2022.63.4.4

[6] Moore E.M., Lockwood D.N. 2010. Treatment of visceral leishmaniasis. *Journal of Global Infectious Diseases* 2(2): 151–158. doi:10.4103/0974-777X.62883

[7] Lockard R.D., Wilson M.E., Rodríguez N.E. 2019. Sex-related differences in immune response and symptomatic manifestations to infection with *Leishmania* Species. *Journal of Immunology Research* 2019: 4103819. doi:10.1155/2019/4103819

[8] Helou D.G., Mauras A., Fasquelle F., Lanza J.S., Loiseau P.M., Betbeder D., Cojean S. 2021. Intranasal vaccine from whole *Leishmania donovani* antigens provides protection and induces specific immune response against visceral leishmaniasis. *PLoS Neglected Tropical Diseases* 15(8): e0009627. doi:10.1371/journal.pntd.0009627

[9] Pradhan S., Schwartz R.A., Patil A., Grabbe S., Goldust M. 2022. Treatment options for leishmaniasis. *Clinical and Experimental Dermatology* 47(3): 516–521. doi:10.1111/ced.14919

[10] de Jesus J.A., Laurenti M.D., Antonangelo L., Faria C.S., Lago J.H.G., Passero L.F.D. 2021. Related pentacyclic triterpenes have immunomodulatory activity in chronic experimental visceral leishmaniasis. *Journal of Immunology Research* 2021: 6671287. doi:10.1155/2021/6671287

[11] Olías-Molero A.I., de la Fuente C., Cuquerella M., Torrado J.J., Alunda J.M. 2021. Antileishmanial drug discovery and development: time to reset the model?. *Microorganisms* 9(12): 2500. doi:10.3390/microorganisms9122500

[12] Al-HAlbosiy M.M.F., Ali H.Z., Hassan G.M., Ghaffarifar F. 2020. Artemisinin efficacy against old world *Leishmania donovani*: *in vitro* and *ex vivo* study. *Annals of Parasitology* 63(3): 295–302. doi:10.17420/ap6603.267

[13] Hassan G.M., Ali H.Z. 2020. *Ex vivo* study of anti-leishmanial activity of artemisinin against *Leishmania tropica* amastigote. *Research Journal of Pharmacy and Technology* 13(8): 3787–3787. doi:10.5958/0974-360x.2020.00670.8

[14] Al Musayeib N., Mothana R., Gamal A., Al-Massarani S., Maes L. 2013. *In vitro* antiprotozoal activity of triterpenoid constituents of *Kleiniaodora* growing in Saudi Arabia. *Molecules* 18(8): 9207–9218. doi:10.3390/molecules18089207

[15] Nikiéma J.B., Vanhaelen-Fastré R., Vanhaelen M., Fontaine J., De Graef C., Heenen M. 2001. Effects of antiinflammatory triterpenes isolated from *Leptadeniahastata* latex on keratinocyte proliferation. *Phytotherapy Research* 15(2): 131–134. doi:10.1002/ptr.700

[16] Das A., Jawed J.J., Das M.C., Parveen S., Ghosh C., Majumdar S., Saha B., Bhattacharjee S. 2021. Lupeol and amphotericin B mediate synergistic anti-

leishmanial immunomodulatory effects in *Leishmania donovani*-infected BALB/c mice. *Cytokine* 137: 155319. doi:1016/j.cyto.2020.155319

[17] Sadiq M. Al□Hamash. 2012. Study of visceral leishmaniasis (kala-azar) in children of Iraq. *Mustansiriya Medical Journal* 11(2): 15–19.

[18] Neamah S.D., Ali H.Z., Al-Halbosiy M.M.F. 2023. Artemisinin efficacy on iNOS production in U937 cell-line infected with *Leishmania donovani*. *Iraqi Journal of Science* 46(2): 525–535. doi:10.24996/ijs.2023.64.2.3

[19] Bates P.A. 1994. Complete development cycle of *Leishmania mexicana* in axenic culture. *Parasitology* 108: 1–9. doi:10.1017/s0031182000078458

[20] Hassan G.M., Ali H.Z. 2019. Leishmanicidal activity of artemisinin against cutaneous leishmaniasis, *in vitro*. *Journal of Biotechnology Research Center* 13(1): 16–22. doi:10.24126/jobrc.2019.13.1.563

[21] Das A., Jawed J.J., Das M.C., Sandhu P., De U.C., Dinda B., Akhter Y., Bhattacharjee S. 2017. Antileishmanial and immunomodulatory activities of lupeol, a triterpene compound isolated from *Sterculia villosa*. *International Journal of Antimicrobial Agents* 50(4): 512–522. doi:1016/j.ijantimicag.2017.04.022

[22] Alvar J., den Boer M., Dagne D.A. 2021. Towards the elimination of visceral leishmaniasis as a public health problem in east Africa: reflections on an enhanced control strategy and a call for action. *Lancet. Global Health* 9(12): e1763–e1769. doi:10.1016/S2214-109X(21)00392-2

[23] Colares A.V., Almeida-Souza F., Taniwaki N.N., Taniwaki N.N., Souza C.F., Martins da Costa J.G., Calabrese K., Abreu-Silva A.L. 2013. *In vitro* antileishmanial activity of essential oil of *Vanillosmopsis arborea* (Asteraceae) Baker. *Evidence-Based Complementary and Alternative Medicine* 2013: 727042. doi:10.1155/2013/727042

[24] Machado M., Pires P., Dinis A.M., Santos-Rosa M., Alves V., Salgueiro L., Cavaleiro C., Sousa M.C. 2012. Monoterpenic aldehydes as potential anti-*Leishmania* agents: activity of *Cymbopogon citratus* and citral on *L. infantum, L. tropica* and *L. major*. *Experimental Parasitology* 130(3): 223–231. doi:10.1016/j.exppara.2011.12.012

[25] Schmidt T.J., Khalid S.A., Romanha A.J., Alves T.M., Biavatti M.W., Brun R., Da Costa F.B., de Castro S.L., Ferreira V.F., de Lacerda M.V., Lago J.H., Leon L.L., Lopes N.P., das Neves Amorim R.C., Niehues M., Ogungbe I.V., Pohlit A.M., Scotti M.T., Setzer W.N., de N C Soeiro M., Steindel M., Tempone A.G. 2014. The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases – part II. *Current Medicinal Chemistry* 19(14): 2176–2228.

[26] Chan-Bacab M.J., Peña-Rodríguez L.M. 2001. Plant natural products with leishmanicidal activity. *Natural Products Rep*ort 18(6): 674–688. doi:10.1039/b100455g

[27] Sen R., Chatterjee M. 2011. Plant derived therapeutics for the treatment of leishmaniasis. *Phytomedicine* 18(12): 1056–1069. doi:10.1016/j.phymed.2011.03.004

[28] Machado V.R., Sandjo L.P., Pinheiro G.L., Moraes M.H., Steindel M., Pizzolatti M.G., Biavatti M.W. 2018. Synthesis of lupeol derivatives and their antileishmanial and antitrypanosomal activities, *Natural Product Research* 32(3): 275–281. doi:10.1080/14786419.2017.1353982

[29] Hoet S., Pieters L., Muccioli G.G., Habib-Jiwan J.L., Opperdoes F.R., Quetin-Leclercq J. 2007. Anti-trypanosomal activity of triterpenoids and sterols from the leaves of *Strychnos spinosa* and related compounds. *Journal of Natural Products* 70(8): 1360–1363. doi:10.1021/np070038q

[30] Kaur G., Chauhan K., Kaur S. 2019. Lupeol induces immunity and protective efficacy in a murine model against visceral leishmaniasis. *Parasitology* 146(11): 1440–1450. doi:10.1017/S0031182019000659

[31] Saha S., Profumo E., Togna A.R., Riganò R., Saso L., Buttari B. 2020. Lupeol counteracts the proinflammatory signalling triggered in macrophages by 7-keto-cholesterol: new perspectives in the therapy of atherosclerosis. *Oxidative Medicine and Cellular Longevity* 2020: 1232816. doi:10.1155/2020/1232816

[32] Sen N., Das B.B., Ganguly A., Mukherjee T., Tripathi G., Bandyopadhyay S., Rakshit S., Sen T., Majumder H.K. 2004. Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *Cell Death Differ* 11(8): 924–936. doi:10.1038/sj.cdd.4401435

Received 03 April 2024
Accepted 12 June 2024