Leishmanicidal and apoptotic activities of oleuropein on *Leishmania major*

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**Abstract.** Background: Leishmania is a unicellular protozoan parasite causing a wide range of human diseases ranging from localized self-healing cutaneous lesions to fatal visceral infections. Objective: The aim of the present study is to assess the cytotoxic, anti-proliferative, and apoptotic effects of oleuropein on *Leishmania major* promastigotes (MHOM/SA/84/JISH) and to compare its effects with the reference drug sodium stibogluconate (pentostam). Methods: Cytotoxicity and promastigote proliferation were measured using MTT colorimetric assay. Furthermore, the Annexin V/propidium iodide staining technique followed by flow cytometry was used for studying the cell death properties of oleuropein. Results: In the present report we have shown that oleuropein, a pharmacologically safe, natural product of olive leaf, has a potent leishmanicidal effect. Indeed, oleuropein exhibits cytotoxic and anti-proliferative effects against *Leishmania major* promastigotes. Moreover, oleuropein triggers death through apoptosis, whereas pentostam induces death mainly via necrosis on *Leishmania major* promastigotes. Conclusion: Here we demonstrate for the first time that the non-toxic, natural product oleuropein has apoptotic properties against *Leishmania major* promastigotes. Further studies are needed to investigate its molecular pathway.

**Introduction**

Leishmaniasis is a vector-borne protozoan disease caused by species of protozoan parasites of the genus Leishmania, which are intracellular parasites of the mononuclear phagocyte system [1, 2]. Leishmania species are responsible for parasitic diseases with a wide range of symptoms and considerable impact on public health worldwide. The disease is widespread, affecting 12 million people around the world, with about 1 – 2 million estimated new cases occurring every year [3]. Transmission to the vertebrate host, initiated by flagellated metacyclic promastigotes, occurs via the bite of an infected female sandfly vector. Once within the mammalian host, the parasite enters the macrophage, where it transforms into the replicative amastigote stage [4]. There are various forms of local treatment modalities for cutaneous leishmaniasis, such as heating, freezing, curettage, and infiltration with antimonial agents, which have their own advantages and disadvantages [5]; however, the drugs most commonly used to treat leishmaniasis are pentavalent antimonials, amphotericin B, paromomycin, and pentamidine, which require high dosage over long periods and are administered parenterally [6]. One of the commonly used pentavalent antimonials is sodium stibogluconate (pentostam), which has serious side effects [7]. To overcome the complications caused by leishmaniasis chemotherapy, an effective drug would be valuable if it offers a safe treatment at a reasonable cost. Phytotherapy has recently received considerable attention as an alternative to chemotherapy in parasitic disease control. Oleuropein is the most abundant of the phenolic compounds in olives [8]. Several studies have shown that oleuropein possesses a wide range of pharmacologic and health-promoting properties, including anti-atherogenic[9], antiviral [10], antimicrobial [11], hypotensive [12], and antidiabetic effects [13].

To further explore for the antiparasitic properties of oleuropein, we investigated its leishmanicidal and antiproliferative effects on *Leishmania major* promastigotes; moreover, we studied the apoptotic pathway underlying its effects on *Leishmania major* promastigotes.

**Key words**
oleuropein – *Leishmania major* – cytotoxicity – apoptosis
Materials and Methods

Parasite culture

*Leishmania major* promastigotes (MHOM/SA/84/JISH) of Saudi strains were cultured in complete medium consisting of RPMI-1640 (GIBCO, City?, Federal State? USA) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM NaHCO3, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Sigma Aldrich, City?, Federal State?, USA). Incubation of parasites was carried out at 26°C in 5% CO2. Promastigotes were harvested and used for the evaluation of antileishmanial activity of drugs.

Cell viability measurements by MTT assay

Different concentrations of oleuropein (Sigma Aldrich, USA) or the reference drug sodium stibogluconate (pentostam) (GlaxoSmithKline, City?, UK) in Roswell Park Memorial Institute (RPMI) media were prepared. Exponential-phase *L. major* promastigotes in cultured media (1.5 × 10⁶/mL) were seeded into 96-well plate and treated with the desired concentrations of the drugs at final concentrations of 50, 100, 200, 250 µg/mL. Control wells were left without treatment, blank wells contained only media. All tests were performed in triplicates. Plates were incubated at 26°C in 5% CO2 for 48 h. A modified MTT colorimetric assay was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Aldrich, USA) for detection of promastigote viability. MTT reagent (250 µg/mL) was added to each well, plates were incubated for 4 h at 26°C. DMSO (DMSO) was added to dissolve the formazan crystals. The amount of cleaved tetrazolium salts to formazan, which directly correlates to the number of metabolically active cells in the culture, was quantified using enzyme-linked immunosorbent assay (ELISA) reader at 540 nm of absorbance [14]. Cell viability was calculated using the equation:

\[ \text{Absorbance of treated sample/absorbance of control cells} \times 100. \]

All values are means of triplicate wells. The 50% inhibitory concentration (LC50), i.e., the drug concentration that decreases the rate of cell growth by 50%, was calculated from the dose responsive curve, and the results were expressed as the means and standard deviations of three independent experiments.

Cell proliferation measurements by MTT assay

Promastigotes were cultured and incubated as in viability assay mentioned above, oleuropein or pentostam was added to the 96-well plates at a final concentration of 50 µg/mL and 40 µg/mL, respectively. Plates were incubated at 26°C in 5% CO2 for different time intervals (0, 24, 48, 72 h). MTT reagent was added, and absorbance was measured using ELISA reader at 540 nm of absorbance.

Apoptosis analysis by Annexin V

Promastigotes were cultured as mentioned above. Cells were either treated with DMSO and used as control or challenged with oleuropein and Pentostam at concentrations of 50, 100, 250 µg/mL. After 48 h, the cells were harvested and washed twice in cold PBS. Promastigotes were stained with propidium iodide (PI) and Alexa Fluor 488 Annexin V. (Life technologies, City?, Germany), according to the manufacturer’s instructions. The percentage of cells was determined by the FACS cadibur apparatus and the Cell Quest Pro software from Becton Dickinson (San Jose, CA, USA). Three independent experiments were performed for each promastigote culture.

Statistical analysis

Data were presented as mean and standard error mean or standard deviation using SPSS-17 statistical software. Comparisons between groups were performed by student’s t-test.
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**Results**

**Oleuropein has cytotoxic effect on *L. major* promastigotes**

The means of the promastigotes survival percentages of both oleuropein and the reference drug (pentostam) were compared statistically using different concentrations of both agents (Table 1). Using MTT cytotoxicity assay, the results showed that promastigotes were highly sensitive to oleuropein. The median lethal concentration of oleuropein (LC50) was 50 µg/mL, while Pentostam showed LC50 of 40 µg/mL, which was comparable to that of oleuropein (Figure 1). At 50 µg/mL, oleuropein showed promastigote survival of 47.1% compared to 40.4% in pentostam, with significant difference between them (*p < 0.01*). The promastigote survival dropped sharply to 22.8% at 100 µg/mL oleuropein, but in the case of pentostam it was only 29.7%, and the difference was significant (*p < 0.05*). Using 200 µg/mL oleuropein, promastigote survival reached 15.4%, while it was 25.8% with pentostam, with a significant difference between them (*p < 0.01*). The promastigote survival dropped to 11.5% by using 250 µg/mL oleuropein compared to only 25.2% in the case of pentostam, and the difference was highly significant (*p < 0.001*) (Table 1) (Figure 1).

**Oleuropein inhibits *L. major* promastigotes proliferation**

We investigated the effect of oleuropein (50 µg/mL) and pentostam (40 µg/mL) on promastigote cell proliferation action using the MTT cell proliferation assay (Figure 2). Results showed that while the control non-treated promastigotes continued proliferating in a time dependent manner, the number of those treated with oleuropein decreased sharply after only 24 h of treatment, with a highly significant difference from the control (0 h) promastigotes (*p < 0.001*) and continued to decrease gradually after 48 h, and continued declining after 72 h of treatment. Compared to the control (0 h), Pentostam also showed inhibition of promastigote proliferation after 24 h of treatment (*p < 0.05*). After 48 h of treatment, the act of inhibition of pentostam on promastigote proliferation increased and continued until after 72 h of

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Table 1. Cytotoxic effects of different concentrations of oleuropein and pentostam on *L. major* promastigotes using MTT assay.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Group</th>
<th>N</th>
<th>Mean % promastigote survival ± Standard deviation</th>
<th>Standard error mean</th>
<th>t</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>0 µg/mL</td>
<td>Pentostam</td>
<td>3</td>
<td>100.0000 ± 1.00000</td>
<td>0.57735</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oleuropein</td>
<td>3</td>
<td>100.0000 ± 1.00000</td>
<td>0.57735</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µg/mL</td>
<td>Pentostam</td>
<td>3</td>
<td>40.4133 ± 1.47852</td>
<td>0.85363</td>
<td>-4.652</td>
<td>0.010**</td>
</tr>
<tr>
<td></td>
<td>Oleuropein</td>
<td>3</td>
<td>47.1367 ± 2.02003</td>
<td>1.16627</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg/mL</td>
<td>Pentostam</td>
<td>3</td>
<td>29.7300 ± 2.52014</td>
<td>1.45500</td>
<td>3.570</td>
<td>0.023*</td>
</tr>
<tr>
<td></td>
<td>Oleuropein</td>
<td>3</td>
<td>22.8467 ± 2.19163</td>
<td>1.26534</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 µg/mL</td>
<td>Pentostam</td>
<td>3</td>
<td>25.8033 ± 2.48760</td>
<td>1.43621</td>
<td>6.065</td>
<td>0.004**</td>
</tr>
<tr>
<td></td>
<td>Oleuropein</td>
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<td>15.3500 ± 1.65058</td>
<td>0.95296</td>
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<td>250 µg/mL</td>
<td>Pentostam</td>
<td>3</td>
<td>25.2267 ± 1.95247</td>
<td>1.12726</td>
<td>8.949</td>
<td>0.001***</td>
</tr>
<tr>
<td></td>
<td>Oleuropein</td>
<td>3</td>
<td>11.5133 ± 1.79804</td>
<td>1.03810</td>
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</table>

Values are given as means ± SD. Values are significant at ***p < 0.001, **p < 0.01, *p < 0.05.**
treatment (Table 2). Comparing the promastigotes growth inhibition of both oleuropein and pentostam, oleuropein has more pronounced effect only after 24 h of treatment, however, its inhibitory effect was almost the same as that of pentostam after 48 h and 72 h of treatment. (Table 2) (Figure 2).

**Oleuropein triggers apoptosis in L. major promastigotes in a dose-dependent manner**

In order to study whether the mechanism of cell death triggered by oleuropein is via apoptosis or necrosis, the Annexin V/propidium iodide (PI) staining technique followed by flow cytometry was used. L. major promastigotes were treated with different concentrations of oleuropein or pentostam for 48 h and then were stained and sorted. Figure 3A shows four groups of cells; viable cells that excluded both Annexin V and PI (Annexin V-/PI-), bottom left; early apoptotic cells that were only stained with Annexin V (Annexin V+/PI-), bottom right; late apoptotic cells that were stained with both Annexin V and PI (Annexin V+/PI+), top right; and necrotic cells that were only stained with PI (Annexin V-/PI+), top left. The proportion of apoptosis was considered as the sum of both early and late apoptosis after deduction of the proportion of spontaneous apoptosis. Figure 3A and B confirmed the cytotoxicity of oleuropein against L. major promastigotes. Importantly, oleuropein triggered death by apoptosis only (Figure 3B), while pentostam triggered death mainly by necrosis (Figure 3C). This effect increased in a dose-dependent manner with a higher effect in oleuropein. At 50 µg/mL, both oleuropein and pentostam showed similar apoptotic effects on L. major promastigotes (17.8% and 18.5%, respectively) without noticeable necrosis (Figure 3C). Oleuropein (100 µg/mL) triggered apoptosis in ~ 60% of promastigotes, while only 10% were apoptotic in response to the same dose in the case of pentostam. The same dose of pentostam (100 µg/mL) triggered necrosis in around 26% of the cells (Figure 3C). Oleuropein (250 µg/mL) induced apoptosis that reached 85.7% compared to only 31.7% in pentostam; however, using the same dose, pentostam showed

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time (hours)</th>
<th>N</th>
<th>Mean proliferation ± Standard deviation</th>
<th>Standard error mean</th>
<th>t</th>
<th>p-value</th>
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<td><strong>Control</strong></td>
<td>0</td>
<td>3</td>
<td>0.3538 ± 0.03005 ± 0.01735</td>
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<td></td>
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<td>24</td>
<td>3</td>
<td>0.4375 ± 0.03524 ± 0.02035</td>
<td>−3.129</td>
<td>0.035*</td>
<td></td>
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<tr>
<td></td>
<td>48</td>
<td>3</td>
<td>0.6812 ± 0.08000 ± 0.04619</td>
<td>−6.635</td>
<td>0.003**</td>
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<tr>
<td></td>
<td>72</td>
<td>3</td>
<td>1.2908 ± 0.16611 ± 0.09590</td>
<td>−9.614</td>
<td>0.001***</td>
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<tr>
<td><strong>Pentostam</strong></td>
<td>0</td>
<td>3</td>
<td>0.3159 ± 0.04036 ± 0.02330</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>24</td>
<td>3</td>
<td>0.1332 ± 0.06182 ± 0.03569</td>
<td>4.287</td>
<td>0.013*</td>
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<tr>
<td></td>
<td>48</td>
<td>3</td>
<td>0.0272 ± 0.00873 ± 0.00504</td>
<td>12.110</td>
<td>0.00***</td>
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<tr>
<td></td>
<td>72</td>
<td>3</td>
<td>0.0236 ± 0.00210 ± 0.00121</td>
<td>12.529</td>
<td>0.00***</td>
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<tr>
<td><strong>Oleuropein</strong></td>
<td>0</td>
<td>3</td>
<td>0.5118 ± 0.02021 ± 0.01167</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>24</td>
<td>3</td>
<td>0.0349 ± 0.00474 ± 0.00274</td>
<td>39.790</td>
<td>0.00***</td>
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<tr>
<td></td>
<td>48</td>
<td>3</td>
<td>0.0291 ± 0.00267 ± 0.00154</td>
<td>41.013</td>
<td>0.00***</td>
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<tr>
<td></td>
<td>72</td>
<td>3</td>
<td>0.0286 ± 0.00110 ± 0.00064</td>
<td>41.356</td>
<td>0.00***</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as means ± SD. Values are significant at ***p < 0.001, **p < 0.01, *p < 0.05.
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35% of necrosis, while oleuropein exhibited only 1% (Figure 3C).

**Oleuropein triggers apoptosis in *L. major* promastigotes in a time-dependent manner**

*L. major* promastigotes were treated with 250 µg/mL oleuropein or pentostam for different time intervals (Figure 4A, B), the apoptotic effect of oleuropein was started at 24 h of treatment (30.5%), and the maximum proportions of cell death (81.5%) were reached after 48 h of treatment (Figure 4A, B). Pentostam showed 11.1% of death at 24 h of treatment, and the rate increased to 30.2% at 48 h. Oleuropein treated promastigotes showed only very little necrosis in all time periods, but pentostam showed high percentages of necrosis of ~ 25% and 38% at 36 h and 48 h of treatment, respectively (Figure 4A, C).

**Discussion**

Most of the drugs currently being used for leishmaniasis suffer from various limits like high toxicity, difficulty in management or development of resistance [15]. Therefore, there is an urgent need for new, safe, more effective, and economically feasible drugs for the treatment of leishmaniasis. The Mediterranean diet, rich in fruits, vegetables, and fish, has been associated with a lower incidence of diseases and an overall improvement in health. These findings were attributed to the high consumption of olive oil and olive leaves (Olea europaea L. Ole-
In the present report, we present clear evidence that oleuropein, the major active element in olive oil and leaf extracts, could constitute a potential therapeutic agent for *Leishmania major* promastigotes in vitro.

The results show that oleuropein has a dose-dependent cytotoxic effect on *L. major* promastigotes (Figure 1). Using 250 µg/mL oleuropein, promastigotes showed survival of 11.5% compared to only 25.2% in the case of pentostam (Table 1). The median lethal concentration (LC50) was 50 µg/mL for oleuropein, which was comparable to that of pentostam (40 µg/mL). In acute toxicity studies for oleuropein, no lethality or adverse effects were observed in mice, even when it was administered at doses as high as 1,000 mg/kg, thus a LD50 could not be determined [16, 17]. The available antileishmanial drugs still rely on the highly toxic pentavalent antimonials, meglumine antimoniate (Glucantime), and sodium stibogluconate (Pentostam), which cause serious side effects and require long-term treatment [7, 18]. Moreover, oleuropein showed inhibition of promastigote proliferation in a time-dependent manner. The inhibition was very sharp after only 24 h of treatment, however, pentostam showed mild decrease in cell proliferation after 24 h compared to oleuropein (Figure 2). At 48 h of treatment, both drugs showed the same inhibitory effect, which was sustained for up to 72 h of treatment. Using high doses of oleuropein in vivo could be safe as it has been confirmed in many other studies [16, 17, 19]; however, pentostam is known for its toxicity and serious side effects in long-term high-dose therapy [20, 21]. Only few studies have

![Figure 4](image)

**Figure 4.** A: Oleuropein triggers apoptosis in *L. major* promastigotes in a time-dependent manner. *L. major* promastigotes were treated with oleuropein or pentostam 250 µg/mL for the indicated periods of time, and cell death was analyzed using the Annexin V/PI flow cytometry assay. Charts indicating the proportion of apoptotic and necrotic cells. *(u no * in Figure!?)TR = top right; BR = bottom right; TL = top left. B: Histogram showing the proportions of apoptotic cells. C: Histogram showing the proportions of necrotic cells. Error bars represent standard deviations of at least three different experiments.
been done showing the antiparasitic effects of oleuropein. One of those studies showed that pure oleuropein extract and oleuropein 60% mixture exhibited leishmanicidal activity against L. major, L. infantum, and L. donovani promastigote stationary phase, with more pronounced effect against L. donovani promastigotes. The authors reported that oleuropein showed activity against stationary, but not logarithmic phase promastigotes on L. major, however, it exhibited growth inhibition of both phases of L. infantum and L. donovani promastigotes [22]. In the present study, oleuropein showed growth inhibition of logarithmic phase promastigotes. The discrepancy in results could be explained by the fact that, leishmania promastigotes are characterized by species heterogeneity found at the ultrastructural, biochemical, genetic, and biological levels [23]. This heterogeneity may lead to variation in sensitivity to several drugs among promastigotes. Leishmania sensitivity to several drugs may vary due to biochemical and molecular differences between species [7]. It was also found that the virulence of leishmania strain and its capacity for growth and adaptation in culture can alter the clonal composition of the strain over successive passages in the medium and so modify the expression of resistance phenotype [24]. Oleuropein was found as proliferation inhibitor for intracellular L. donovani amastigotes, and, in vivo, it exerted depletion of L. donovani in both liver and spleen of infected balb/c mice [22]. In T. gondii tachyzoites, oleuropein showed reduced cell proliferation in a dose-dependent manner. Moreover, it has anti-Toxoplasma gondii activity in vitro by inhibiting proliferation of Madin-Darby bovine kidney cells infected with tachyzoites and inhibiting 55.4% the expansion of parasites in the peritoneal cavity of infected mice [25].

To confirm the cytotoxic nature of oleuropein and to identify the death pathway that this agent triggers in L. major promastigotes, the Annexin V/propidium iodide (PI) staining technique was conducted. Mechanisms of programmed cell death in Leishmania species are activated in response to various chemotherapeutic stimuli such as sodium stibogluconate (pentostam), amphotericin B and meglumine antimonate (Glucantime) as well as nutrient deprivation [26]. At 250 µg/mL, oleuropein induced high cytotoxicity in L. major promastigotes (11% survival), a result which correlates well with the high percentage of apoptosis (86%) obtained using the same concentration; however, pentostam showed only 31.7% apoptosis at 250 µg/mL concentration (Figure 3 A, B). Interestingly, the oleuropein-induced cell death in L. major promastigotes is mainly through apoptosis, compared to pentostam, which triggered cell death mainly through necrosis (Figure 3 A, C). The mechanism by which oleuropein induced apoptosis in L. major promastigotes is unknown, however, under a variety of stress conditions, Leishmania species display some morphological and biochemical features characteristic of mammalian programmed cell death or necrosis [27]. It was previously found that the process of apoptosis shares many similar features in both metazoan and protozoan organisms [28]. Two prominent features observed in both of these eukaryotes are DNA condensation and fragmentation as well as externalization of phosphatidylserine [29]. The apoptotic effect of oleuropein on promastigotes started as early as 24 h of treatment, and the maximum proportions of death were reached after 48 h of treatment (81.5%). Moreover, oleuropein showed only 1.4% of necrotic death (Figure 4 A, B). The percentage of promastigote apoptosis and necrosis shown by pentostam at 48 h of treatment were 30.2% and 38.3%, respectively (Figure 4 A, C). It was previously found that oleuropein and its metabolite hydroxytyrosol have apoptotic effects on L. major, L. infantum and L. donovani promastigotes through programmed cell death [22]. The apoptosis mechanisms in L. major promastigotes are still not clear. It was mentioned in a recent study that Endoplasmic reticulum stress-induced apoptosis in Leishmania major is dependent on the release of Endonuclease G from mitochondria to nucleus via cytoplasm [30]. Using biophysical assays to study the interaction between oleuropein and membrane lipids, strong oleuropein effects on anionic phospholipids such as phosphatidylglycerol were found, which may account for its antimicrobial effects [31]. Oleuropein was also reported to have good anti-granuloma, anti-apoptosis, anti-necrosis effects in T. gondii-infected mice [25].
Collectively, these data show that oleuropein has potent leishmanicidal and antiproliferative properties against *L. major* promastigotes. It triggers cell death mainly through apoptosis and thereby warrants further investigation for its potential use as a chemotherapeutic agent against cutaneous leishmaniasis. We recommend future studies on intracellular amastigotes and animal models. Molecular studies should be conducted for more investigation of the apoptotic pathway of this molecule.

**Conflict of interest**

The authors declared that there were no conflicts of interest.

**Acknowledgments**

This work was supported by the National Plan for Science and Technology (NPST), funded by King Abdulaziz City for Science and Technology (KACST) through project number 10-ENV993-02.

**References**


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