Anti-leishmanial activities of *Olea europaea* subsp. *laperrinei* extracts

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**ABSTRACT**

Leishmaniasis is an infectious disease that is often fatal in affected patients and represents a major public health problem. At present, no vaccine is available, and the drug treatments used are costly, long, and have numerous side effects, they also present variable effectiveness, frequent relapses, and a more and more marked resistance towards the parasites. Thus, new therapeutic strategies are urgently needed, and they are mainly based on the research of active natural products. The objective of our study is the chemical characterization and the quantification of the polyphenol contents contained in the EAF and EAT extracts of the Laperrine olive tree and the evaluation of their antileishmania effect against *Leishmania infantum*. The quantification of polyphenols, flavonoids and total tannins shows a higher content in the leaf extract. We find respectively 776.76±30.64 mg gallic acid equivalent/g DR; 114.35±14.12 mg quercetin equivalent/g DR and 214.89±.17 mg tannic acid equivalent/g DR. The chemical characterization of *Olea europaea* subsp. *laperrinei* extracts show the presence of numerous antileishmanial biomolecules such as oleuropein, hydroxytyrosol, rutin, gallic acid, caffeic acid, rosmarinic acid, and quercetin. In this context, we are testing the *in vitro* leishmanicidal effect of Laperrine olive tree extracts. The results obtained are promising and highlight the effectiveness of the tested extracts against the promastigote form of *Leishmania infantum*. Indeed, the LD50 is obtained with the leaf extract at a concentration of 7.52±2.71 µl/ml.

**Introduction**

Leishmaniasis is an infectious disease transmitted by vectors. A flagellate-bearing protozoan of the Trypanozomatidae family, Leishmania, causes it. The sand fly is the vector of the disease, and only the female is haematophagous(1). It transmits the infection to humans during a blood meal by injecting the parasite in its extracellular, flagellated promastigote form (Figure 1A). In the epidermis, the promastigotes are phagocytosed by macrophages, which surround them with a vacuole containing dehydrogenases. As these enzymes are not specific, the parasite escapes this cellular defense (2) and assumes the amastigote form in the macrophage (Figure 1B). After the parasite multiplies and the macrophage bursts, the amastigotes locally infect new phagocytic cells and migrate to other tissues.

The clinical expression and course of the disease depend on both the tropism of the Leishmania species involved and the human immune response (3). According to Alvar et al. (4) and Lecoeur et al.(5), infection causes severe immunopathologies in humans and animals that can be fatal. In immunocompromised individuals, leishmaniasis considered an opportunistic disease and is associated with human immunodeficiency virus (HIV) infection(6).

In the majority of cases, the infection is asymptomatic; it leads to cutaneous lesions characteristic of cutaneous leishmaniasis (CL), which are confined to the skin. When lesions involve the ENT sphere (nose, mouth) and dermis, this is called muco cutaneous leishmaniasis (MCL) (7).

Visceral leishmaniasis (VL) affects the internal organs of the host. The entire reticulo-histiocytic system, spleen, and internal lymph nodes are affected. VL is fatal if left untreated(8). According to Solano-Gallego et al. and Messahel et al. (2,9) the parasite responsible for Mediterranean foci is *Leishmania infantum*. In Algeria, *Phlebotomus perniciosus* (Figure 1C) is recognized as the vector of this infection (10).

Currently, there is no vaccine for humans and only a few treatment options are available(13). These have many disadvantages in terms of their relative efficacy and toxicity, leading to cardiac, hepatic, nephrological, and pancreatic complications (14). In addition, they are associated with high costs, and the long duration of treatment leads to
the emergence of parasite resistance (15,6).

The active substances contained in the olive tree are of growing scientific interest for the development of new therapeutic molecules and represent an alternative to toxic chemicals. Olive leaves are known to be rich in biophenols, which have both nutritional and therapeutic properties (16). These compounds possess antimicrobial (17,18) and antiprotozoal properties (19). The bioinsecticidal activity of olive extracts proved to be effective against the mealybug (20) and against the olive psyllid (21).

*Olea europaea* subsp. *laperrinei* (Batt. et Trab.) (Figure 2A) is a subspecies of the olive tree in the family *Oleaceae*. It is endemic and naturally adapted to the dry areas of Hoggar, Tefedest, Tassili N’ajjer, and Mouyedir (Algerian Central Sahara).

**Materials and Methods**

**Plant material**

Harvesting of the petioles used in our experiment is done on adult mother plants at Ouled Hanghassi station (23°14’50.1”N, 5°29’13.7”E) in Tamanrasset region in May 2021. The olive tree is identified by its characteristics using (22). The harvested leaves are separated from the stems, sorted, and then dried in the open air, in the shade and protected from moisture. They are then ground separately (particle diameter between 0.5 and 1 mm) and stored in the dark at room temperature. The powder obtained is used for the aqueous extractions. The various analyzes began during the same year.

**Biological material**

The strain of *Leishmania infantum* responsible for VL, which was tested in our trials, belongs to the cryobank of the Parasite Ecodepilogerie Service of the Institute Pasteur (Algeria). It is the strain LIPA 1227, listed under the code MON1/DZ/01/LIPA1227/01.

**Extraction procedure**

Dissolve 20 g of plant powder in 200 ml of distilled water. After maceration for 24 hours under stirring (100 rpm) in the dark and at room temperature, the extracts are filtered on Whatman paper No. 1 and the filtrate is freeze-dried. The dry residues obtained are stored at 4°C. By weighing the residues, the yield of each extract can be determined.

**Determination of total phenol content**

Total phenols are determined according to the Folin-Ciocalteu method described by Singleton and Rossi (23). The content of total polyphenols is determined by extrapolation to the standard curve obtained from a series of dilutions of gallic acid (100 μg/ml). To each test tube, add 0.25 ml of the sample to be determined, 1.25 ml of Folin-Ciocalteu (1/10), and 1 ml of sodium carbonate (75 g/l). Then the absorbance is measured at 765 nm. The content of total phenols is given in milligrams of gallic acid equivalent per gram of dry residue (mg GAE/g DR).

**Determination of the total flavonoid content**

The method used is the same as that described by Chang et al. (24). To 0.5 ml of the solution of each extract, add 1.5 ml of methanol, 0.1 ml of aluminium chloride (10 %), 0.1 ml of potassium acetate (1 M), and 2.8 ml of distilled water.

The mixture is incubated for 30 minutes at room temperature. The absorbance is measured at 415 nm. The standard curve is established with quercetin (100μg/ml) and the linear regression equation allows the calculation of the total flavonoid content expressed as milligram quercetin equivalent per gram plant powder (mg EQ/g DR).

**Determination of total tannin content**

The determination of tannins is carried out according to the protocol developed by Hagerman and Butler (25). 1 ml of BSA solution (1 mg/ml) is mixed with 500 μl of the extract solution. After incubation at 4°C for 24 hours, the precipitate is collected by centrifugation at 750 g for 15 minutes at 4°C and then dissolved in 2 ml of sodium dodecyl sulphate (SDS)/triethanolamine (TEA) (1%/5%) to which 500 μl of FeCl3 reagent has been added. After incubation for 15 minutes, the absorbance is measured at 510 nm.

The total tannin content, expressed as milligrams of tannic acid equivalent per gram of dry residue (mg EAT/g DR), is determined from the tannic acid calibration curve using the linear regression equation.

**Chemical composition**

The chemical composition of the aqueous extracts of leaves (EAF) and stems (EAT) is obtained by heating in the liquid phase under the following operating conditions: Quantitative analysis is carried out using an AGILENT 1100 series liquid chromatograph equipped with a diode array detector (DAD) with several wavelengths selected according to the maximum absorptions of the molecules sought, a quaternary pump, an on-line degasser, and an automatic injector. The chromatograph is equipped with a Hypersil BDS -C18, 5um, 250X4.6 column at 30°C. The mobile phase consists of water acidified with 0.2 % acetic acid at a pH of 3.1 and acetonitrile in a linear elution gradient over 30 minutes at 1.5 ml/min, starting with 95 % H2O and ending with 100 % acetonitrile. The injected volume corresponds to 5 μl of the extract dissolved in methanol at 0.16 g/ml. The percentage of each chemical compound in the extract is determined from the peak areas obtained. The phenolic compounds are identified by comparing the retention times of the different standards analyzed with the...
A factorial correspondence analysis (FCA) was performed with a 5% risk. It aimed to deal with several variables at the same time in order to establish correlations between them.

**Ethical Approval**

The proposed study has been reviewed by the Ethics and Deontology Committee of Mouloud Mammeri University, Tizi-Ouzou, Algeria. The corresponding ethical approval code is UMMTO/25/01/2022/Eth-Deon-A-102.

**Results**

**Quantitative analysis**

To characterize the aqueous extracts of leaves (EAF) and stems (EAT) of the Laperrine olive tree, a quantitative study is carried out using colorimetric methods (folin-ciocecalteu, aluminium trichloride and formation of the protein-tannin complex).

The total content of phenolic compounds is determined using the respective standard curves. The results obtained reveal an obvious variability in the calculated contents. In deed, the statistical analysis shows a significant difference between the mean values in the case of total phenols (P = 2.71 \times 10^{-6}) where we obtained a value of 776.76±30.64 mg EAG/g DR for EAF and 94.45±1.64 mg EAG/g DR for EAT, and in the case of total flavonoids (P = 3.56 \times 10^{-6}), with a calculated mean value of 114.35±14.12 mg EQ /g DR for EAF and 24.92±0.95 mg EQ /g DR for EAT. For total tannins, the calculated means correspond to 214.89±20.17 mg EAT /g DR for EAF and 90±1.47 mg EAT /g DR (P = 0.0004).

**Qualitative analysis**

The chemical composition of the two extracts was determined by high-performance liquid chromatography using the same procedure and by their UV spectra (20).

**Table 1. Main chemical compounds identified in the extracts of the Laperrine olive tree.**

<table>
<thead>
<tr>
<th>Chemical compounds</th>
<th>EAF extract retention time (min.)</th>
<th>Area (%)</th>
<th>EAT extract retention time (min.)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>3.329</td>
<td>0.61</td>
<td>3.903</td>
<td>0.36</td>
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<tr>
<td>Hydroxyquinone</td>
<td>3.748</td>
<td>1.79</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Hydroxytyrosol</td>
<td>4.885</td>
<td>1.27</td>
<td>4.871</td>
<td>1.75</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>5.987</td>
<td>1.06</td>
<td>5.540</td>
<td>0.58</td>
</tr>
<tr>
<td>Catechol</td>
<td>6.371</td>
<td>0.87</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catechin</td>
<td>-</td>
<td>-</td>
<td>6.176</td>
<td>0.48</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>7.051</td>
<td>2.34</td>
<td>7.053</td>
<td>1.96</td>
</tr>
<tr>
<td>Aesculetin</td>
<td>7.056</td>
<td>1.14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>7.188</td>
<td>1.39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Orientin</td>
<td>7.984</td>
<td>2.20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rutin</td>
<td>8.966</td>
<td>0.77</td>
<td>8.933</td>
<td>3.85</td>
</tr>
<tr>
<td>Quercetin</td>
<td>9.228</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O Anisic acid</td>
<td>9.531</td>
<td>1.13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Luteolin 7 Glucoside</td>
<td>-</td>
<td>-</td>
<td>9.490</td>
<td>4.15</td>
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<tr>
<td>Salicylic acid</td>
<td>9.968</td>
<td>2.28</td>
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<td>-</td>
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<td>Rosmarinic acid</td>
<td>10.238</td>
<td>1.84</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Naringenin 7 Glucoside</td>
<td>10.504</td>
<td>2.10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Apigenin 7 Glucoside</td>
<td>-</td>
<td>-</td>
<td>10.525</td>
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<td>Oleuropein</td>
<td>11.269</td>
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<td>11.249</td>
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<td>m Anisic acid</td>
<td>11.789</td>
<td>2.44</td>
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<td>-</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>13.631</td>
<td>2.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>14.984</td>
<td>1.40</td>
<td>15.067</td>
<td>6.15</td>
</tr>
</tbody>
</table>
Anti-leishmania activity

The results obtained show that both extracts of Laperrine olive have antileishmanial activity. The increase in this activity is proportional to the concentration of the extracts. However, there is a significant difference \( (P=3.5610^{-6}) \) between EAF and EAT. Exposure of the parasite to a maximum concentration \((100 \mu g/ml)\) of the extract results in a percentage viability of \(36\%\) for the EAF extract and \(50.33\pm0.58\%\) for the EAT extract (Figure 3). Moreover, our results show that the extracts of Laperrine olive are more efficient in eliminating promastigotes than the positive control \(T^+\) corresponding to Glucantime, for which we record an average value of \(68\pm2\%\) at the therapeutic dose. For the negative control \(T^-\), the percentage of viability is \(100\%\).

Anti-Leishmania activity, expressed by parasite viability, is strongly correlated with the concentrations of the extracts tested. The trend curves applied to the graphs in Figure 4 shows a very high linear fit coefficient for the extract EAF, which is 0.95. In other words, more than 95% of the viability observed in the period considered is explained by the effect of the leaf extract. We, therefore, assume that the extract is probably more effective than the extract of EAT, in eliminating the parasite. However, we note that the effect of the stem extract is not negligible.

From the resulting regression equations, the 50% lethal dose \((LD50)\) of the extracts EAF and EAT is determined. Thus, for the leaf extract \(Y = -0.133 X + 49; R^2 = 0.95\), a dose of \(7.52 \pm 2.71 \mu g/ml\) is sufficient to cause the death of \(50\%\) of the parasites. For the stem extract \(Y = -0.100 X + 59.29; R^2 = 0.81\), the concentration required to eliminate \(50\%\) of the promastigotes is on average 12 times higher than that of the extract from EAT.

To explain the effect of the extracts on the viability of \(L. \text{ infantum}\), we integrated their chemical composition to interpret the results and correlate the different variables. The data were subjected to a factorial correspondence analysis (FCA) and are shown in Figure 5.

On axis 1 of the AFC (Figure 5), a negative correlation is observed between EAF extract and LD50. It appears that a group of 18 molecules correlated with this set would explain the particular effect of EAF extract on the viability of the parasite studied.

These molecules are gallic acid (GA), hydroxyquinone (HQ), hydroxytyrosol (HY), resorcinol (Res), catechol (Cal), vanillic acid (AV), aesculin (Aes), caffeic acid (CA), orientin (Ori) rutin (Rut), quercetin (Que), o anisic acid (OAA), salicylic acid (SA), rosmarinic acid (RA), rosmarinic acid (RA), naringenin 7 glucoside (7G Nar), oleuropein (Ole), m anisic acid (MAA) and cinnamic acid (CiA).

Since the extract of EAF has a non-zero effect on the viability of promastigotes, we can say that the molecules common to both extracts, namely gallic acid, hydroxytyrosol, resorcinol, vanillic acid, rutin, and oleuropein, are the main ones responsible for eliminating 50% of the parasites.

Discussion

A study by Katanilic et al. (28), in which 70 medicinal plants were classified according to their richness in total phenols quantified from an aqueous extract, showed that the extracts of Laperrine olive are very rich in polyphenols. Comparison of our results with those of Nashwa et al. (29) from a methanolic extract of \(Olea \text{ europaea}\) leaves, which had a value of \(90.48\pm0.16\) mg EAG/g residue, shows that the polyphenol content in Laperrine olive leaves is very high.

Phytochemical analysis carried out on the two extracts (HPLC). We identified 22 chemical molecules, 19 in the EAF extract and only 10 in the extract from EAT (Table 1).

Our results indicate the presence of hydroxytyrosol and oleuropein in the extracts, compounds typical of \(Olea \text{ europaea}\). Quercetin, rutin, vanillic acid, salicylic acid, aesculetin, cinnamic acid, and caffeic acid are found in \(Olea \text{ europaea}\) leaves (21). Moreover, our results are similar to those obtained by Lahcene et al. (20) after acid hydrolysis and Djenane et al. (27) with a methanolic extract of Laperrine olive leaves.
tracts revealed the presence of two important fractions in phenolic compounds, namely flavonoids and tannins, whose contents are particularly high. This abundance seems to be a response of the species to the conditions of its environment and clearly shows the close relationship between the biosynthesis of secondary metabolites and the extreme environmental conditions to which the Laperrine olive tree is exposed. It should be noted, however, that compared to the extract of EAT, the content of phenols and total flavonoids in the EAF extract is significantly higher.

The total flavonoid content of Laperrine olive leaves is higher than that calculated by Abaza et al. (30) from *Olea europaea* leaves of the Chetoui variety, where they found an average of 6.23 ± 0.62 mg EC /g dry weight for an aqueous extract and 15.83 ± 1.26 mg EC /g dry weight for an ethanolic extract. For Botsoglou et al. (31), the content calculated from an ethanolic extract corresponds to 12.46 ± 0.58 mg EC /g dry weight of *Olea europaea* L. leaves.

The total tannin content is higher in the leaves of Laperrine olive than in the leaves of *Olea europaea* L., corresponding to a value of 79.70 mg EC /100g dry weight for the Chemlal variety and a value of 73.05 mg EC /100g dry weight for the Neb jmel variety, Neb jmel, 2013. However, the tannin content of *Olea laperrinei* is close to 22.79 g EAT /kg DM, a value determined by Mebiourou-Boudedich and et al. (32) for the leaves of *Olea europaea*.

The results obtained in the antileishmanial test are consistent with the work of Sifaoui et al. (33) and Koutsoni et al. (34), who highlighted an inhibitory effect of polyphenols and flavonoids against the promastigote form of the parasite. According to Kheirandish et al. (7), phenolic structures damage parasite membranes. The application of Laperrine olive leaf extract on promastigotes had better results than *Vitis vinifera*, *Arbutus unedo*, and *Myrtus nivellei* leaf extract (Table 2).

The scientific literature confirmed our results. Indeed, the work of Ribeiro et al. (38), Belmonté-Reche et al. (39), Koutsoni et al. (34), and Ghafouri et al. (40) have demonstrated the involvement of hydroxytyrosol, oleuropein, gallic acid, and rutin in anti-leishmanial activity.

However, the greater efficacy of EAF extract compared to EAT extract could be explained by the exclusive presence of caffeic acid, rosmarinic acid, and quercetin. These molecules, according to Carter et al. (41), inhibit arginase, which is important for the multiplication and infectivity of the parasite. On the other hand, hydroxyquinone, catechin, acsulin, orientin, O anisic acid, salicylic acid, naringenin 7 glucoside, malic acid, and cinnamic acid, which could play a synergistic role in enhancing the effect studied.

**Conclusion**

As far as we know, no study has yet been conducted on the antiparasitic effects of Laperrin olive. Therefore, the results presented in this report show for the first time the efficacy of Laperrine olive tree extracts as a potential leishmanicial.

The leishmania-fighting activity demonstrated in vitro is directly related to the phenolic content of the extracts studied and depends on the various interactions (synergy or antagonism) that determine it. Thus, the leishmanicial potential is in favour of the EAF extract, whose phenolic content is higher than that of the EAT extract. The results obtained in our study clearly show that the olive leaf extract from Laperrine has a strong leishmanicial activity against the promastigote form of Leishmanianfaut. This is due to the combined presence of oleuropein, hydroxytyrosol, caffeic acid, rutin, quercetin, gallic acid, and rosmarinic acid, whose anti-leishmanial activity has been demonstrated by many authors.

The richness and diversity of phytochemical molecules contained in the Laperrine olive tree make it a good candidate to serve as a potential source of new biomolecules. It could be used as an alternative to harmful chemicals and as a source of biomolecules with leishmanicial activity. However, purification of the bioactive molecules and separate or combined assessments of their activity is needed to better understand their mechanism of action on the parasite.

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**Institutional Review Board Statement**

The proposed study has been reviewed by the Ethics and Deontology Committee of Mouloud Mameri University, Tizi-Ouzou, Algeria. The corresponding ethical approval code is UMMTO/25/01/2022/Eth-Deon-A-102.

**Informed Consent Statement**

Not applicable.

**Data Availability Statement**

Data is available with the investigators and may be provided upon reasonable request.

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7: (5).


