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Caffeic acid and quercetin exert caspases-independent apoptotic effects on *Leishmania major* promastigotes, and reactivate the death of infected phagocytes derived from BALB/c mice

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ABSTRACT

Objective: To investigate the leishmanicidal effects of two antioxidants, caffeic acid and quercetin on *Leishmania major* (*L. major*) promastigotes *in vitro*, and their immunomodulatory effects on infected phagocytes derived from susceptible BALB/c mice.

Methods: Caffeic acid and quercetin-induced cell death was examined by Pi-Hoechst double staining of *L. major* promastigotes and MTT assay, in the presence or absence of protease inhibitors *in vitro*. Caffeic acid or quercetin were administered subcutaneously to BALB/c mice infected with *L. major* promastigotes through a dorsal air pouch. Nitric oxide and superoxide anion production by phagocytes infiltrating the air pouch and the expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B in the air pouch membrane were therefore evaluated using appropriate methods.

Results: Caffeic acid and quercetin displayed a dose-dependent cytotoxic effect against *L. major* promastigotes, and induced cell death *via* caspases-independent pathways. *In vivo*, *L. major* promastigotes inoculation into air pouch cavity of BALB/c mice leads to a sequential influx of neutrophils (hours), followed by macrophages (days). Results showed that *L. major* delayed apoptosis of infected neutrophils and macrophages by the cleavage of the nuclear factor kappa B $p65^{RelA}$ subunit, and persisted by inhibiting TNF-α and iNOS expression and reactive oxygen species generation. Caffeic acid or quercetin restored reactive oxygen species production and TNF-α-induced iNOS activity, and abrogate apoptosis delay of infected phagocytes.

Conclusions: The leishmanicidal effect of caffeic acid and quercetin on promastigotes and amastigotes, as well as reactivation of infected phagocytes apoptosis, suggested a potential therapeutic role against cutaneous leishmaniasis.

1. Introduction

Algeria is the second largest focus of cutaneous leishmaniasis, and one of the eight countries that cumulate 90% of cutaneous leishmaniasis, worldwide [1]. *Leishmania infantum* and *Leishmania major* (*L. major*) are the causative agents of two endemic clinical forms of cutaneous leishmaniasis in northern and southern Algeria, respectively [2,3].

Neutrophils are innate cells that internalize *Leishmania* promastigotes in the first hours of infection, before being themselves phagocytised by macrophages, about two days later [4,5]. This silent way of infection reinforces the role of neutrophil in innate response to leishmaniasis.

Intracellular oxidative killing of phagocytised pathogens by neutrophils involves nicotinamide adenine dinucleotide phosphate oxidase 2 activation and rapid and massive production of reactive oxygen species (ROS) by oxidative burst. In addition, neutrophils can form extracellular traps (NETs), a network of double strands of DNA fragments coated with antimicrobial molecules that contribute to the extracellular killing of parasites [6,7]. NETs participate to cell death by both ROS dependent and independent pathways [8].

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All experiments were conducted in accordance with the Ethical Committee for animals' welfare of the Université des Sciences et de la Technologie Houari Boumediene, Algiers, Algeria.

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Leishmania parasite shows some sensitivity to superoxide anion ($O_{2.-}$), derived from the respiratory burst of phagocytes, primarily neutrophils [9–11]. However, it can escape oxidative attack by the inhibition of oxidative burst, and the blockade of early fusion of tertiary and specific granules with the parasite-containing phagosome [12,13].

Nitric oxide (NO) produced by interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) activated macrophages is the most effective endogenous oxidant for killing intracellular amastigotes [14]. Parasite killing is also dependent on neutrophil elastase and TNF-α production in resistant B6 mice. Otherwise, the phagocytosis of apoptotic neutrophils exacerbates *L. major* growth through prostaglandin E2 and transforming growth factor beta (TGF-β) production by macrophages of BALB/c susceptible mice [15–17].

Infected phagocytes have delayed apoptosis involving nuclear factor kappa B (NF-κB) signalling [18,19]. Thus, deciphering the role of NF-κB in immunity to *L. major* could have crucial implications for the design and delivery of vaccines.

A variety of stress stimuli, such as heat shock and NO can induce an apoptotic-like cell death independently of caspases activation, which leads to fragmentation of genomic DNA into many oligonucleosomes [20–22].

Various therapeutic molecules were used against leishmaniasis with little success, due to their toxicity, chemo-resistance and their high cost. Thus, most people living in affected areas depend largely on popular treatments and traditional medicine practices [23].

Natural compounds such as plant extracts deployed potent immuno-modulatory potential, which could be a convenient adjuvant to conventional chemotherapy.

Phenolic compounds are subdivided into phenolic acids (caffeic acid), flavonoids (quercetin), and tannins. Recent studies demonstrated significant immuno-modulatory effects and anti-leishmanial activity of polyphenols, *in vitro* [24–26].

This study evaluated the apoptotic and leishmanicidal effects of quercetin, and the hydroxy derivative of cinnamic acid, a precursor of flavonoids, caffeic acid on promastigotes *in vitro*, and on the production of superoxide anion, and NO by macrophages and neutrophils from BALB/c mice infected through a dorsal air pouch, as well as the expression of TNF-α, inducible nitric oxide synthase (iNOS) and NF-κB in subcutaneous tissue of apex pouch membrane.

2. Materials and methods

2.1. Reagents

Caffeic acid and quercetin were obtained from Fluka and BDH, respectively. All other chemicals were from Sigma Aldrich, except otherwise indicated.

2.2. Ethics statement

All experiments were conducted in accordance with the Ethical Committee for animals' welfare of the Université des Sciences et de la Technologie Houari Boumediene, Algiers, Algeria.

2.3. Evaluation caffeic acid and quercetin effects on *L. major* promastigotes *in vitro*

2.3.1. Production of promastigotes

Algerian strain of *L. major* (MHOM/DZ/2000/LIPA1126) was kindly provided by the Laboratoire d'Eco-Epidémiologie et Génétique des Populations, Institut Pasteur (Algiers, Algeria). *L. major* promastigotes were collected at stationary phase from biphasic NNN (Novy-McNeal-Nicolle) and RPMI 1640 culture media supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL of penicillin and 100 μg/mL of streptomycin.

2.3.2. Determination of *L. major* promastigotes viability

The effect of caffeic acid and quercetin on promastigotes viability was determined by modified MTT assay [27]. Promastigotes (0.2×10^6 cells/well) in a 96-well plate were treated with increasing concentrations of caffeic acid or quercetin (0, 150, 250 and 400 μmol/L). After 24 h at 25 °C, cell pellets were collected and suspended in 100 μL of phosphate buffer saline (PBS) containing 1 mg/mL MTT, and incubated for 4 h at 25 °C.

Intracellular MTT-formasan crystals were dissolved, and absorbance was read at 550 nm. Otherwise, stationary phase parasites were exposed to a heat shock at 55 °C followed by 1 h incubation at 25 °C.

The % of viable cells was calculated as follows: (AT – AB)/(AC – AB) × 100, where AC, AT, AB were absorbance of control, treated cells and blank, respectively [28].

2.3.3. Determination of *L. major* promastigotes apoptosis

The morphological changes induced by caffeic acid and quercetin were analyzed on promastigotes smears stained with May-Grunwald-Giemsa. Up to 20 microscopic fields were screened under light microscope for each sample (MOTIC®). Otherwise, aliquots of promastigotes were attached on positively charged super frost slides and loaded with the fluorescent nuclear stain propidium iodide (5 μg/mL), and Hoechst 33258 (1 μg/mL) for 30 min. The number of apoptotic cells was evaluated for 100 cells under fluorescence microscopy (Carl Zeiss Inc., Thornwood, NY).

2.3.4. Determination of proteases activity in *L. major* promastigotes

The role of caspases and other proteases in caffeic acid or quercetin induced cell death was examined by treatment of promastigotes (0.2×10^6/200 μL) in a 96-well plate for 2 h with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) or 10 mmol/L ethylenediaminetetraacetic acid (EDTA), and then incubated with increasing concentrations of caffeic acid or quercetin (0, 150, 250 and 400 μmol/L) for 24 h at 25 °C. The viability of promastigotes was evaluated using MTT assay [27].

2.4. Dorsal air pouch infection of susceptible BALB/c mice

BALB/c mice (6–8 weeks old) were bred under controlled conditions. They were supplied standard rodent chow and water, *ad libitum*.

Dorsal air pouches were raised on BALB/c mice ($n = 6$) by subcutaneous injection of 4 mL of sterile air on Days 0 and 3. *L. major* promastigotes (5×10^6/air pouch) were sterilely inoculated at Day 6. Control air pouches received sterile PBS or 2% starch. Treated groups received 25 mg/kg of caffeic acid or quercetin, subcutaneously once daily for 4 days. Mice were lethally anesthetized at 0 h, 4 h, 24 h, and 96 h. Air pouch exudates were collected and analyzed for their leukocytes content.

Apex of pouch membranes were punctured with a scalpel and separated from adjacent subcutaneous and paraspinal tissues. Specimens of apex membranes were either fixed in 10% buffered formalin overnight and processed for histological analyses, or homogenized in 50 mmol/L ice-cold phosphate buffer (pH 7.2) containing 0.5% TritonX-100 and freezed-thawed three times. Homogenates were centrifuged at 10 000 g for 10 min at 4 °C. Membrane supernatants were collected and stored at −70 °C for biochemical analyses.

2.5. Biochemical assays

2.5.1. NO assay

Equal volumes of air pouch exudates or membranes supernatants and Griess reagent (1% sulfanilamide and 0.1% N-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) were incubated for 20 min at room temperature. Absorbance was measured at 540 nm, and nitrites levels were expressed as µmol/L of nitrites per mg of protein.

2.5.2. Superoxide anion assay

Phagocytes harvested at 4 h, 24 h and 96 h post *L. major* inoculation in pouch cavity, were seeded in a 96-well plate at 10^6 cells/mL of PBS pH 7.4, containing 80 µmol/L cytochrome C, and stimulated by 160 nmol/L of phorbol 12-myristate, 13-acetate (PMA) for 30 min at 37 °C [29].

Absorbance was read at 550 nm with a microplate reader (EL800), and the results were expressed as µmol/L/min/10^6 cells.

2.5.3. Assessment of phagocytes recruitment into inflamed tissues

Neutrophils influx was assessed by myeloperoxidase (MPO) activity. Membranes supernatants were added to 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/mL of o-dianisidine hydrochloride. The reaction was started with 0.000 5% H_2O_2, and the absorbance at 460 nm was read after 5 min at 30 °C. MPO activity was expressed as µmol/L of H_2O_2/min/mg protein [30].

Macrophages recruitment was quantified by α-naphtyl acetate esterase activity (ANAE), using a slightly modified method [31]. Briefly, membranes supernatants were incubated in 20 mmol/L sodium phosphate buffer solution (pH 7.0) and 25 mmol/L α-naphtyl-acetate for 15 min at 30 °C. After o-dianisidine treatment, absorbance was read at 600 nm. ANAE activity was expressed as µmol/L α-naphthol min/mg protein.

2.5.4. Assessment of cell death

Thin sections of apex pouch membranes or cell suspensions collected from exudates at 4 h, 24 h and 96 h post infection were exposed to the DNA binding dyes propidium iodure (5 µg/mL) and Hoechst (1 µg/mL), 30 min at room temperature [32]. Cell apoptosis was evaluated on about 100 phagocytes and 200 cells under fluorescence microscopy for nuclear morphology alterations (Carl Zeiss Inc., Thornwood, NY).

2.6. Immuno-histochemical detection of iNOS, TNF-α and NF-κB expression

Histological sections of formalin fixed apex membranes were spread on positively charged super frost slides. Antigen was retrieved by incubating the slides in Tris–EDTA buffer (pH 9.0), 0.05% Tween-20, for 30 min at 95 °C. Endogenous peroxidases and nonspecific binding sites were blocked with 3% H_2O_2 for 10 min followed by 0.3% non-fat dry milk for 2 h.

The slides were incubated overnight at 4 °C with the primary antibody $p65^{RelA}$, anti-iNOS, and anti-TNF-α (1:100, Invitrogen-Life Technologies), and then treated for 2 h with biotinylated goat anti-rabbit immunoglobulin horseradish peroxidase-conjugated streptavidin (1:500), and visualized using 3,3'-diaminobenzidine as substrate (Invitrogen-Life Technologies). The slides were counterstained with hematoxylin, and 20 fields/section were analyzed by light microscopy (MOTIC®). Pictures were taken using a digital camera at 200, 400 and 1000 resolution.

2.7. Statistical analysis

Data were analyzed using student's *t*-test. Differences between infected mice compared to infected treated mice were considered significant at $P < 0.05$. Apoptosis rates were analyzed by Mann–Whitney's *U* test. Two independent experiments were analyzed for five to six mice per group.

3. Results

3.1. Caffeic acid and quercetin induced cell death in *L. major* promastigotes

L. major cell death was investigated by light and fluorescence microscopy (Figure 1A, B) and by MTT assay (Figure 1C).

In contrast to control promastigotes that displayed elongated form, caffeic acid and quercetin treated promastigotes had granular ovoid or round shape morphology with condensed chromatin, and a short and thick flagellum evoking amastigotes (Figure 1A).

As shown in propidium iodure and Hoechst doubled-stained slides, (51 ± 1)% ($P < 0.001$) of caffeic acid-treated promastigotes were apoptotic, compared to 2% of apoptotic untreated cells. The percentage of propidium iodure-stained cells ranged from 2.00% in caffeic acid-treated promastigotes to 29.26% in quercetin-treated promastigotes, indicating that caffeic acid leishmanicidal activity is driven primarily by apoptosis, while quercetin preferentially induced necrosis (Figure 1).

Caffeic acid and quercetin decreased the viability of promastigotes in a dose-dependent manner that reached 68.90% and 59.22%, respectively with the dose of 400 µmol/L.

3.2. Caffeic acid and quercetin induced caspases-independent promastigotes death

Caffeic acid (400 µmol/L) reduced the viability of promastigotes to (31.09 ± 0.45)% of control. This remained unchanged in the presence of PMSF, a serine proteases inhibitor (38.62 ± 1.03)% and EDTA, a metalloproteases inhibitor (42.29 ± 1.24)%. Quercetin (400 µmol/L) reduced the viability of extracellular parasite to (40.77 ± 7.97)% of control. PMSF (44.44 ± 4.56)% and EDTA (47.40 ± 1.99)% were without any further effect (Figure 1C).

Proteases implication in the death of *Leishmania* was also assessed by fluorescence. The level of caffeic acid induced apoptotic promastigotes (51 ± 1)% remained unaltered in

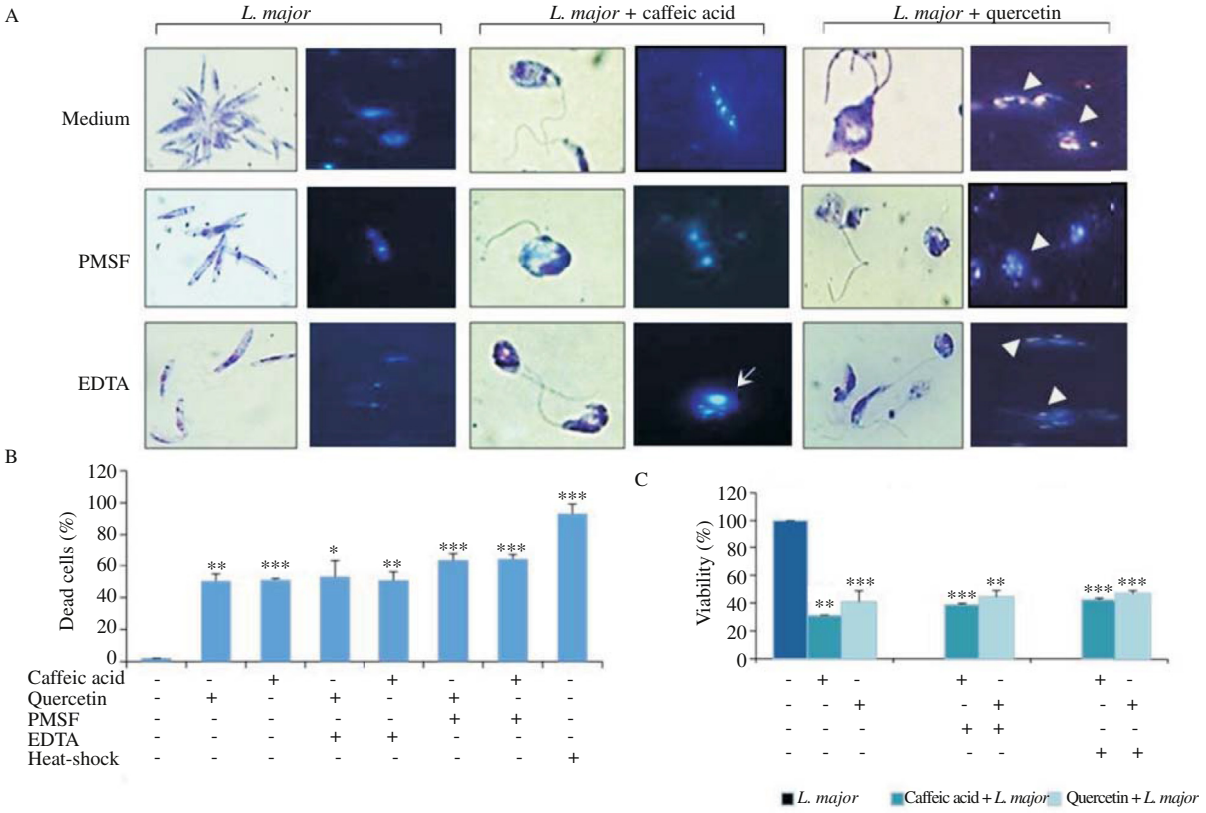

Figure 1. *L. major* cell death is independent of serine proteases and metalloproteases.
A: Promastigotes were treated *in vitro* with or without caffeic acid or quercetin (400 µmol/L) in the presence or absence of protease inhibitors, were stained with May Grunwald Giemsa (left panel) or propidium iodure-Hoechst (right pannel). Treated cells were clearly apoptotic (arrow) or necrotic (arrow head) as shown by their rounded shape and their bright blue or pink staining, respectively; B: Percentage of dead cells (fragmented nuclei) after treatment with caffeic acid or quercetin for 24 h in presence or absence of protease inhibitors; C: Promastigotes were incubated with increasing concentrations of caffeic acid or quercetin (0–400 µmol/L) for 24 h in presence or absence of protease inhibitor, and the MTT assay was performed as described in Methods. Means ± SD were from at least three experiments in triplicate. Unpaired *t*-test, *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

presence of PMSF (63.33 ± 2.88)% and EDTA (50.66 ± 5.50)%. Quercetin induced-cell death (54.33 ± 8.02)% remained unchanged in presence of PMSF (64 ± 4)% and EDTA (53 ± 10.14)%. These results suggested that caffeic acid or quercetin caused a protease-independent programmed cell death of *Leishmania* promastigotes (Figure 1A, B).

3.3. L. major induced leukocytes recruitment, in vivo

Inoculation of *L. major* led to a rapid and massive accumulation of inflammatory cells in air pouch cavity. Neutrophils (77.50 ± 8.18)%, macrophages (18.33 ± 6.65)%, eosinophils (9.00 ± 5.29)%, and lymphocytes (1.33 ± 0.57)% were recruited 4 h after *L. major* inoculation. Neutrophils level was maintained at 82.75%, 24 h post infection (Figure 2).

This infection resulted in a massive influx of leucocytes in pouch membranes (Figure 3A, Dd). They reached (76.50 ± 4.04)% and (77.50 ± 3.53)%, respectively 4 h and 24 h post infection and decreased to (37.00 ± 4.24)% at 96 h (Figure 3B), while the number of macrophages enhanced from (14.50 ± 0.70)% to (52.50 ± 3.53)% at 4 h and 96 h, respectively (Figure 3C, D).

3.4. Caffeic acid and quercetin reduced inflammatory cells infiltrate

Caffeic acid and quercetin decreased neutrophils influx in air pouch cavity by 10.68% and 3% at 4 h ($P < 0.01$), and by 63.74% and 62.13% at 24 h ($P < 0.01$) (Figure 2B). In contrast, neutrophils number enhanced by 21.38% and 25.25% at 4 h ($P < 0.05$), and by 39.62% and 32.17% at 24 h ($P < 0.05$), respectively, compared to infected group (Figure 3C).

L. major enhanced the relative density of macrophages in air pouch exudates by 2.8 and 2.7 fold, and by 7.2 and 7.4 fold at 4 h and 24 h after caffeic acid and quercetin treatment (Figure 2C). However, caffeic acid increased the number of macrophages (2.47, 2.31 and 1.45 fold) at 4 h, 24 h and 96 h, and reduced the rate of neutrophils (1.89 fold) at 96 h in skin of infected mice. No significant difference was observed in the density of resident macrophages of quercetin-treated mice, compared to infected group (Figure 3C).

MPO activity increased by 152% ($P < 0.001$) and 155% ($P < 0.01$) at 4 h and 24 h, while ANAE increased by 112.73% ($P < 0.05$) at 96 h. Caffeic acid and quercetin reduced MPO activity in membranes supernatants by 61.27% and 59.25% ($P < 0.001$), by 51.64% and 41.65% ($P < 0.001$), and by 25% ($P < 0.05$) and 2.74% ($P < 0.05$) at 4 h, 24 h and 96 h, respectively (Figure 4A).

Esterase activity decreased by about 32% with caffeic acid ($P < 0.05$) and quercetin ($P < 0.01$) 4 h after infection, while no significant change was observed at 24 h. Caffeic acid enhanced by 20.57% ANAE activity at 96 h post infection, concomitantly with enhanced macrophages influx (Figure 4B).

3.5. Caffeic acid and quercetin enhanced apoptosis of infected cells

Fluid exudates and pouch membranes of *L. major* infected BALB/c mice contained viable inflammatory cells harbouring

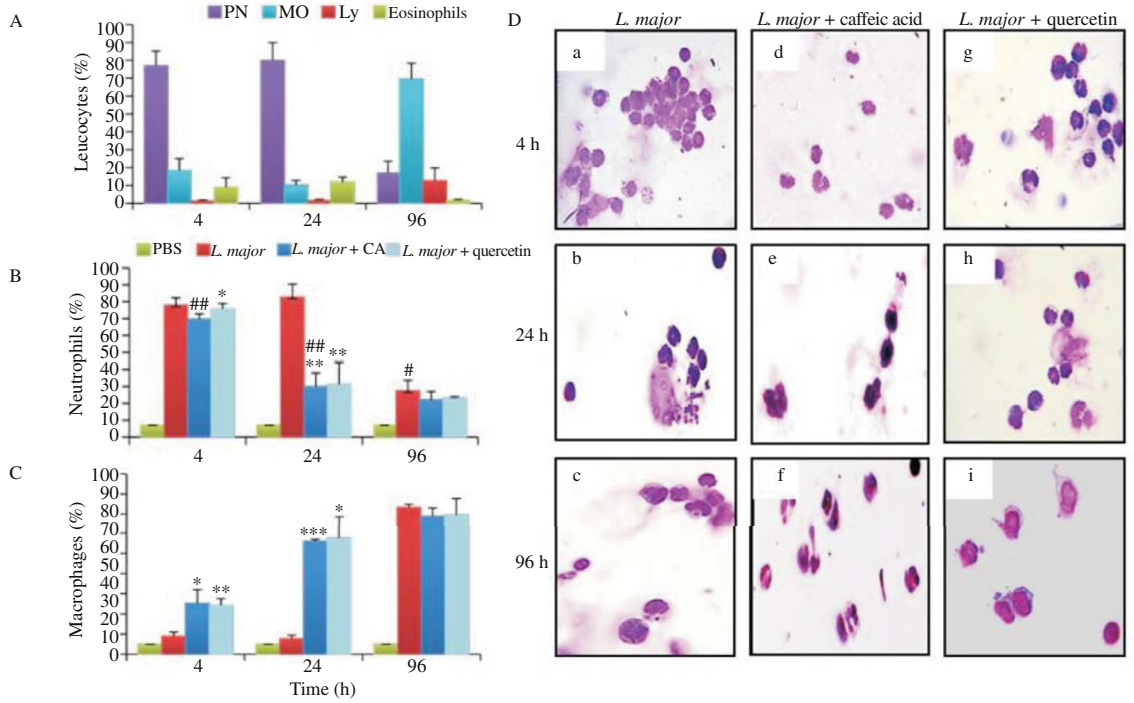

Figure 2. Caffeic acid and quercetin repress cells influx in air pouch cavity.

A: Percentage of neutrophils, macrophages, lymphocytes and eosinophils over total leucocytes in air pouch exudate; B and C: Percentage of neutrophils and macrophages in air pouch exudates of caffeic acid and quercetin treated BALB/c mice. Means ± SD were from three independent experiences, using unpaired *t*-test, *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; Caffeic acid or quercetin treated *v.s.* untreated; $^{\#}$: $P < 0.05$; $^{\#\#}$: $P < 0.01$; $^{\#\#\#}$: $P < 0.01$ infected *v.s.* uninfected cells; D: MGG staining of cells from fluid exudates in *L. major* infected BALB/c. Cells were collected from pouch exudates, centrifuged, and stained with MGG. a: Predominance of neutrophils in *L. major* induced air pouch, 4 h post infection; b, c: Infected neutrophils (arrow head) and macrophages (arrow) at 24 h and 96 h after *L. major* inoculation; d: Reduction of neutrophils number in exudates of caffeic acid treated mice, 4 h after inoculation; e: Cells from air pouch exudates of caffeic acid treated mice, 24 h after infection; f: Phagocytosis of neutrophils by macrophages in caffeic acid treated mice; g: Neutrophils from quercetin treated mice, 4 h after parasite inoculation; h, i: Cells from quercetin treated mice, 24 h and 96 h post infection. Original magnification 400×.

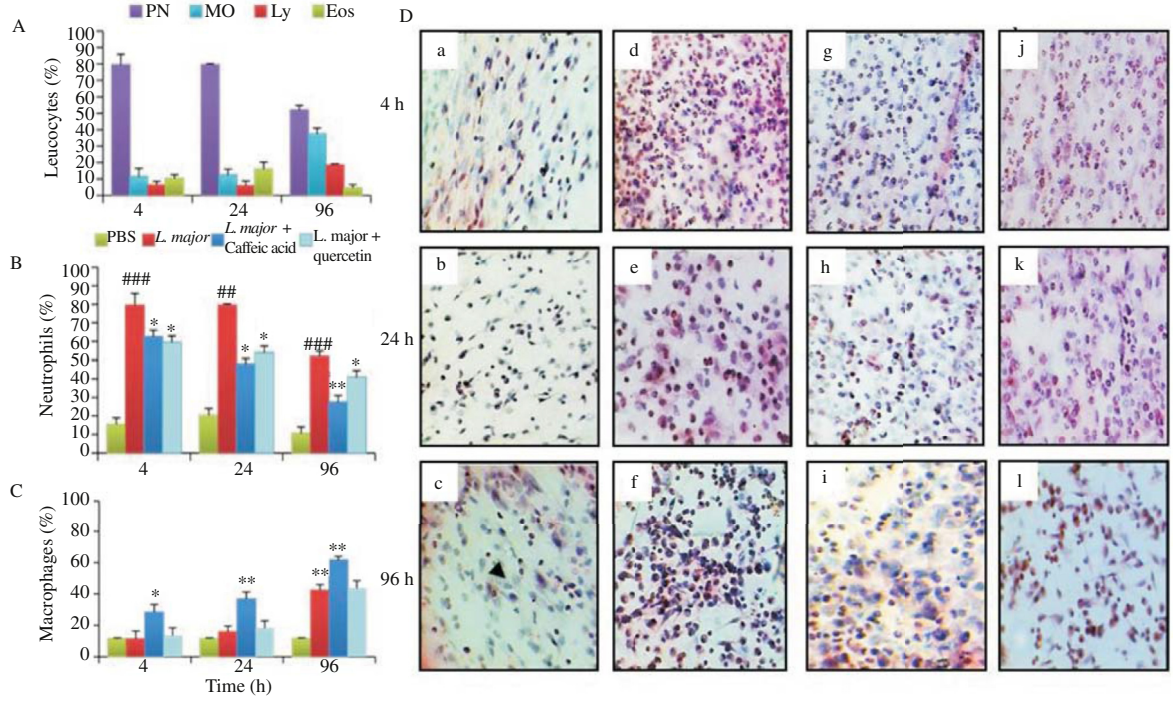

Figure 3. Caffeic acid and quercetin repress cells influx in infected skin.

A: Percentage of neutrophils, macrophages, lymphocytes and eosinophils over total leucocytes in air pouch exudates; B and C: Percentage of neutrophils and macrophages in subcutaneous tissues of caffeic acid and quercetin treated BALB/c mice. Means ± SD were from three independent experiences, using unpaired *t*-test, *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ caffeic acid or quercetin treated *v.s.* untreated cells; $^{\#}$: $P < 0.05$; $^{\#\#}$: $P < 0.01$; $^{\#\#\#}$: $P < 0.01$ infected *v.s.* uninfected cells; D: H&E stained sections of apex membrane from *L. major* infected BALB/c mice. Membranes ($n = 5$) were dissected from the cutaneous tissues, 4 h, 24 h and 96 h post infection; a, b, c: Subcutaneous tissue of BALB/c mice at 4 h, 24 h and 96 h after PBS treatment (Control); d: Predominance of neutrophils 4 h post infection; e: Infected neutrophils (arrow head) and macrophages (arrow) at 24 h; f: Predominance of macrophages at 96 h; g, h, i: Apex pouch membranes in caffeic acid treated mice 4 h, 24 h and 96 h post infection; j, k, l: Apex pouch membrane from quercetin treated mice, 4 h, 24 h and 96 h post infection. Original magnification 400×.

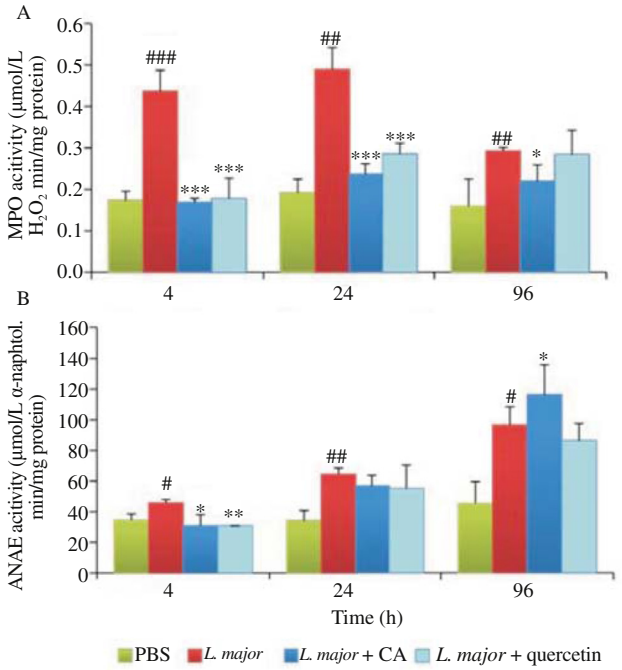

Figure 4. Effect of caffeic acid and quercetin on phagocytes recruitment in subcutaneous tissue of *L. major* infected BALB/c mice.
A: MPO activity assessed in membrane supernatants of BALB/c mice; B: ANAE activity assessed in membrane supernatants of BALB/c mice. Means ± SD were from three independent experiences, using unpaired *t*-test, *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ caffeic acid or quercetin treated *v.s.* untreated cells; $^{\#}$: $P < 0.05$; $^{\#\#}$: $P < 0.01$; $^{\#\#\#}$: $P < 0.01$ infected *v.s.* uninfected cells; CA: Caffeic acid.

viable amastigotes (Figures 5 and 6A). The rate of apoptosis remained unchanged, compared to PBS-treated mice (Figure 6B). Interestingly, caffeic acid and quercetin-treated mice revealed the presence of apoptotic and necrotic neutrophils harbouring dead amastigotes as assessed by their respective bright blue or pink staining (Figures 5 and 6A). Caffeic acid and quercetin enhanced apoptosis by 194% and 80% ($P < 0.05$), and by 165% and 91% ($P < 0.01$) in pouch membranes, by 40% and 36% ($P < 0.01$), and by 57% and 51% ($P < 0.01$) in fluid exudates at 24 h and 96 h, respectively (Figure 6B).

3.6. Caffeic acid and quercetin generated ROS in *L. major* primed neutrophils

Our results showed that the level of superoxide anion release by *L. major* treated and PMA stimulated neutrophils *ex vivo* decreased by 81%, 70% and 64% ($P < 0.001$) at 4 h, 24 h and 96 h, respectively, compared to PMA stimulated cells (Figure 7A, B).

Caffeic acid slightly restored superoxide anion production by *L. major* and PMA stimulated neutrophils by 28% and 12% at 24 h and 96 h ($P < 0.05$), respectively. Similarly, quercetin increased superoxide anion release by 63% ($P < 0.05$) at 4 h, but decreased it by 52% and 22% at 24 h and 96 h (Figure 7B).

3.7. Caffeic acid and quercetin restored iNOS expression and activity via TNF-α stimulation

A parallel increase in NO level (15%, $P < 0.05$), and in the expression of iNOS and TNF-α was measured in infected subcutaneous tissue at 24 h and 96 h (Figures 8 and 9).

Caffeic acid and quercetin potentiated NO level by 25.7% ($P < 0.05$) and 16.7% at 4 h, and by 56.0% ($P < 0.05$) and 11.2% respectively, at 96 h, compared to infected mice (Figure 8C).

Similarly, NO level in pouch exudates of infected mice enhanced by 31.04% and 33.87% at 24 h and 96 h, respectively. Caffeic acid restored NO level in exudates by 44.9% ($P < 0.05$), and 88.34% ($P < 0.05$) at 24 h and 96 h (Figure 8D). Whereas, quercetin restored it by 34.64% and 11.18% at 24 h and 96 h, respectively, compared to infected untreated mice.

Caffeic acid restored in parallel, iNOS and TNF-α expression in apex membranes by 86.34% and 155.43% ($P < 0.01$) at 24 h and 96 h. Quercetin was less effective and increased iNOS and TNF-α expression by 36.58% and 24.43% ($P < 0.05$) at 24 h and 96 h (Figure 9).

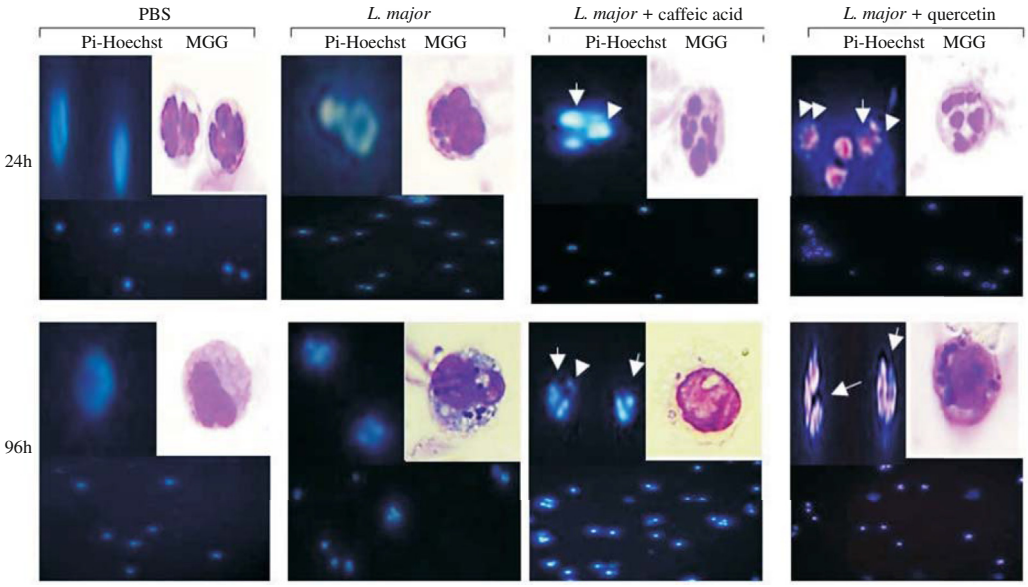

Figure 5. Pro-apoptotic and necrotic effect of caffeic acid and quercetin on innate cells influx 24 h and 96 h post infection.
Propidium iodure-Hoechst and MGG stained sections showed apoptotic or/and necrosis cells containing fragmented nuclei with their pink or bright blue staining, compared to controls. Presence of apoptotic or necrotic neutrophils (arrow) harbouring apoptotic amastigotes (arrow head) in caffeic acid and quercetin treated mice.

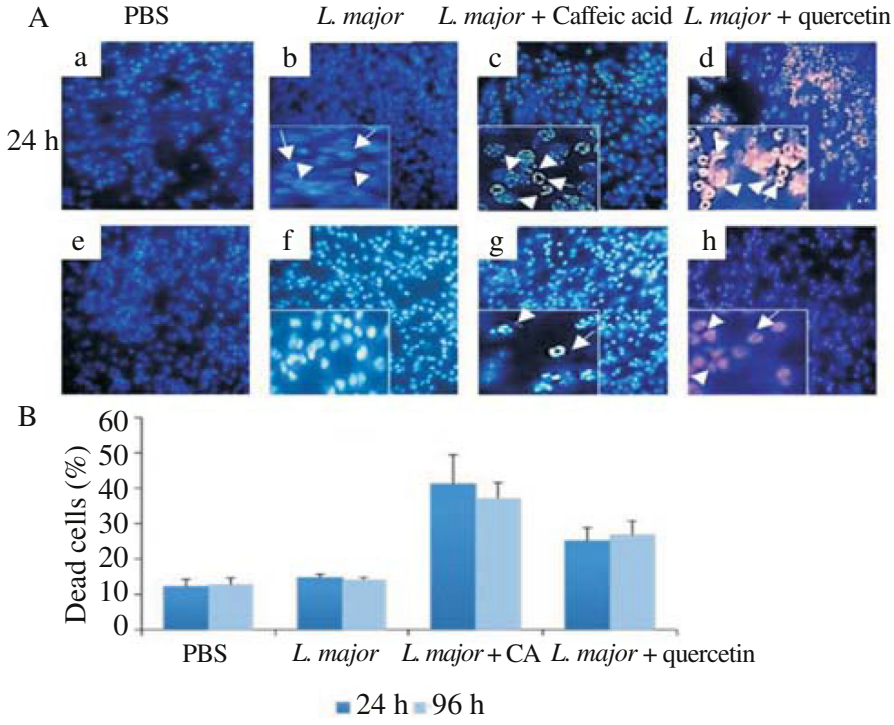

Figure 6. Apoptotic effect of caffeic acid and quercetin on innate cells recruitment in subcutaneous tissue 24 h and 96 h post *L. major* infection.
A: Subcutaneous tissue of dorsal section was harvested at 24 h and 96 h as described in materials and methods section and double stained with Pi-Hoechst; a, e: Subcutaneous tissue of PBS treated BALB/c mice at 24 h and 96 h; b, f: Subcutaneous tissue of *L. major* infected BALB/c mice at 24 h and 96 h showed neutrophils (arrow) harbouring viable amastigotes (arrow head); c, g: Predominance of apoptotic cells (bright blue staining) and presence of apoptotic neutrophils (arrow) harbouring apoptotic amastigotes (arrow head) in caffeic acid treated mice at 24 h and 96 h; d, h: Predominance of necrotic cells (pink staining) in quercetin treated mice at 24 h and 96 h; B: The number of apoptotic cells in Pi-Hoechst stained sections; Cell death was evaluated in subcutaneous tissue of mice treated with caffeic acid or quercetin by observation of 100 cells under fluorescence microscopy (200× and 400×). Means ± SD, using Mann Whitney's *U* test; $^{\#}$: $P < 0.05$ infected *v.s.* PBS-treated mice; *: $P < 0.05$ caffeic acid or quercetin treated *v.s.* untreated mice; CA: Caffeic acid.

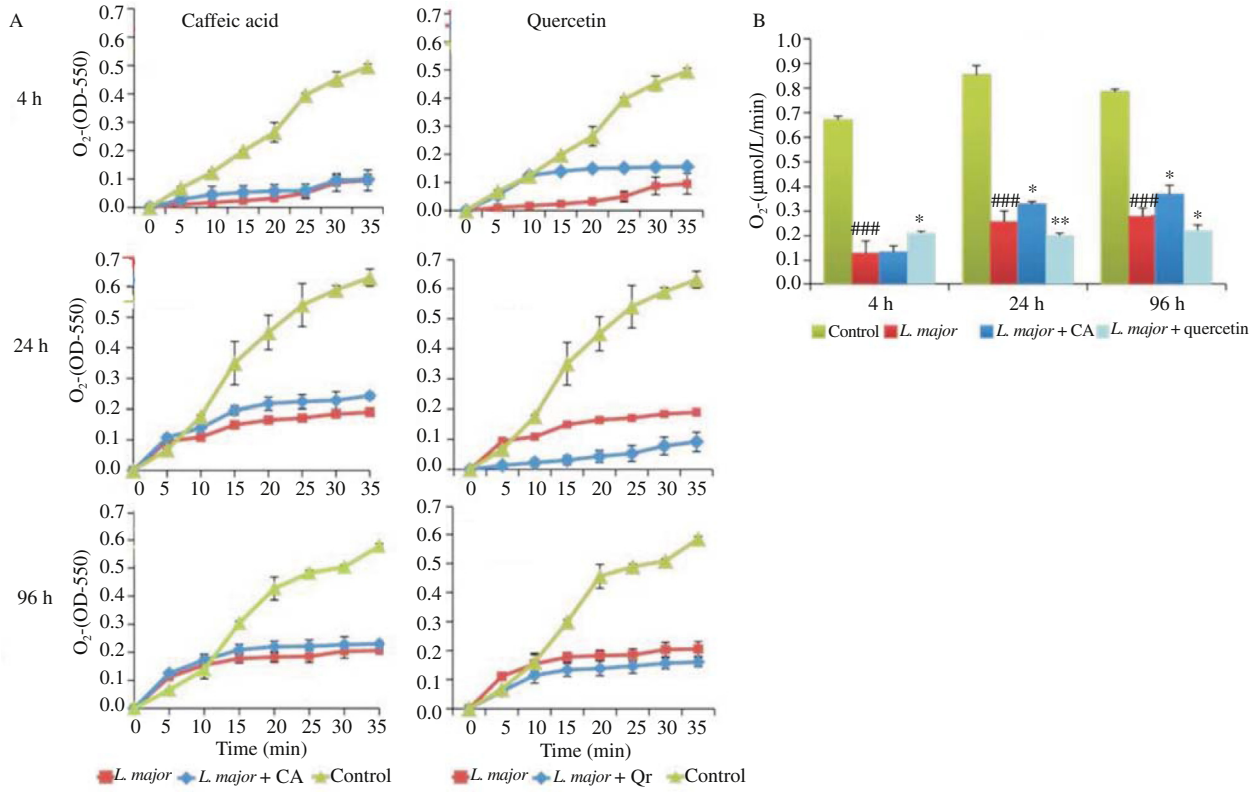

Figure 7. Effect of caffeic acid and quercetin on superoxide anion production by innate cells stimulated by PMA.
A: Kinetic of superoxide anion generation in infected BALB/c mice, 24 h and 96 h after subcutaneous inoculation of 5×10^6 promastigotes into air pouch cavity of caffeic acid or quercetin treated mice; B: The production of superoxide anion in air pouch exudate of caffeic acid and quercetin treated BALB/c mice, recovered after 24 h and 96 h of *L. major* inoculation, and stimulated by PMA. Results were expressed in μmol/L/min/10^6 cells.

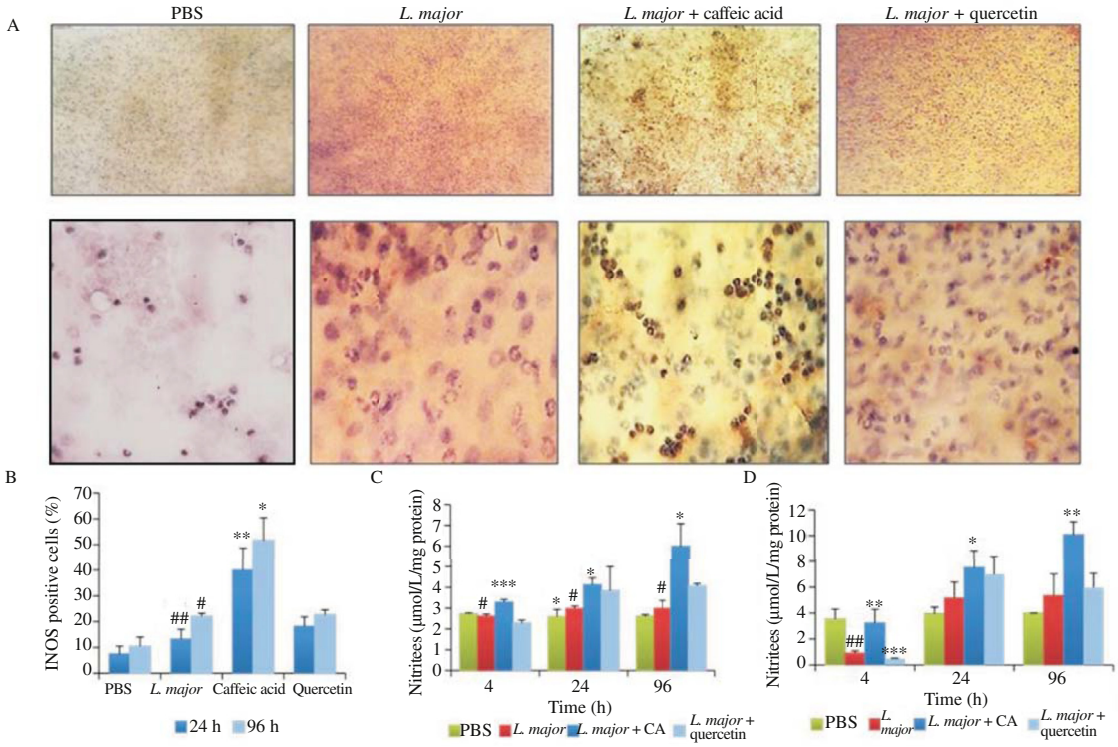

Figure 8. Caffeic acid and quercetin up-regulated iNOS expression in subcutaneous tissue.
A: Representative immuno-histochemical staining of iNOS expression in subcutaneous tissue at 96 h; B: iNOS expression in subcutaneous tissue of BALB/c mice at 24 h and 96 h; C and D: NO production in subcutaneous tissues and exudates; Means ± SD of each experimental group; Significant differences are indicated on graphs, #: $P < 0.05$ infected *v.s.* PBS-treated mice; *: $P < 0.05$ caffeic acid and quercetin treated *v.s.* untreated mice, using Mann Whitney's *U* test; CA: Caffeic acid.

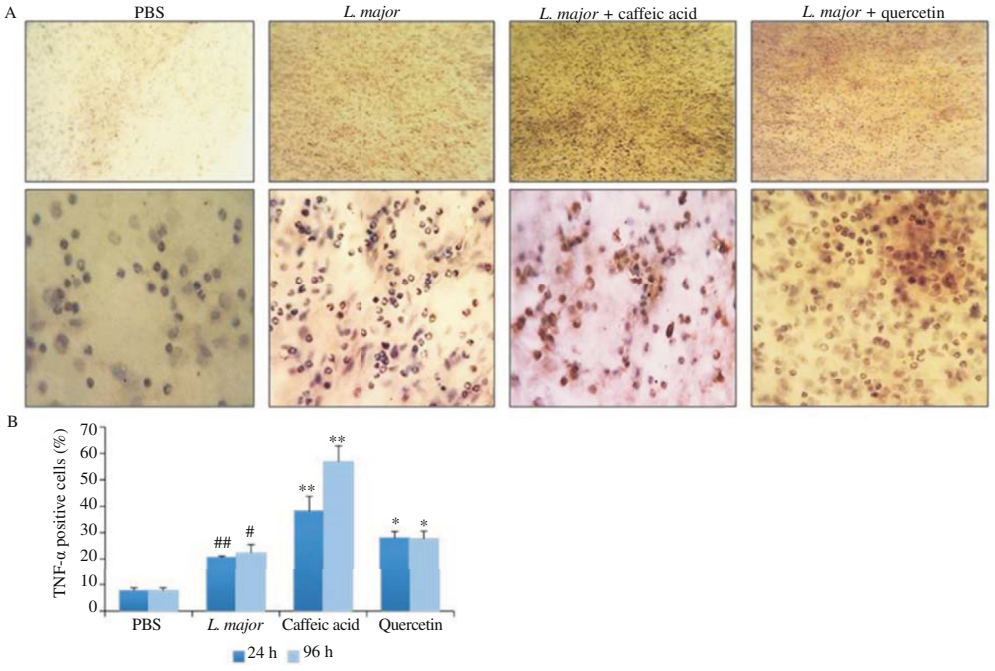

Figure 9. Effect of caffeic acid and quercetin on TNF-α expression in subcutaneous tissue of BALB/c mice at 96 h after *L. major* infection.
A: Representative immuno-histochemical staining of TNF-α expression in subcutaneous tissue of BALB/c mice at 96 h; B: The percentage of TNF-α positive cells in subcutaneous tissue at 24 h and 96 h; Values are means ± SD using Mann Whitney's *U* test; #: $P < 0.05$ infected *v.s.* PBS-treated mice; *: $P < 0.05$ caffeic acid or quercetin treated *v.s.* untreated mice.

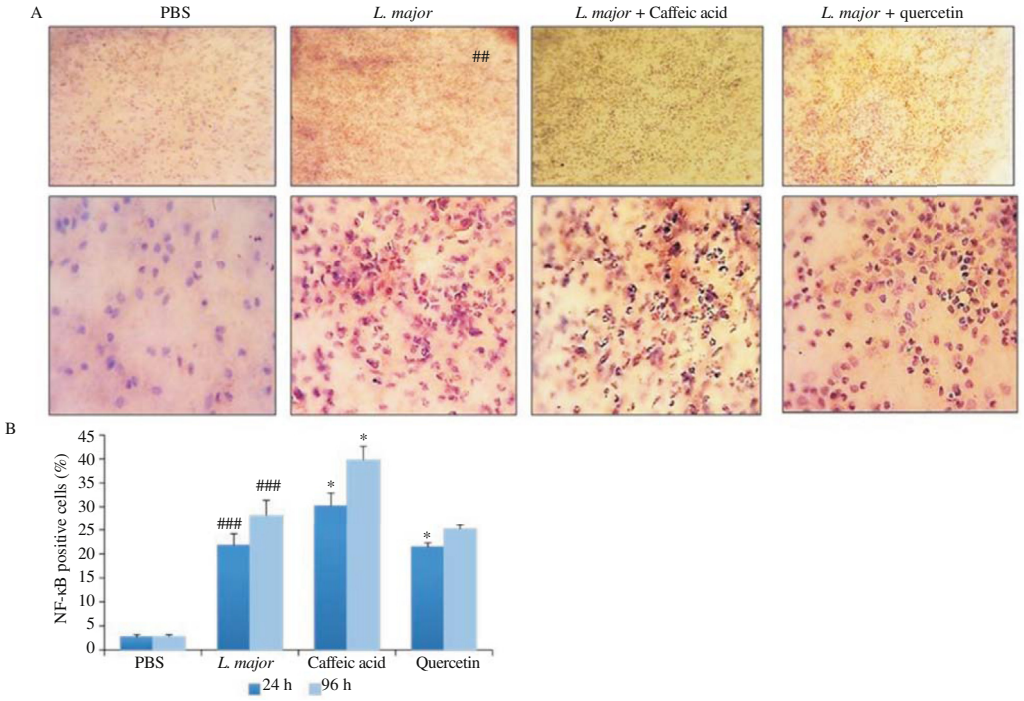

Figure 10. Effect of caffeic acid and quercetin on NF-κB activation in subcutaneous tissue of BALB/c mice at 96 h after *L. major* infection.
A: Representative immuno-histochemical staining of NF-κB expression in subcutaneous tissue of BALB/c mice at 96 h after *L. major* infection; B: The percentage of NF-κB positive cells in subcutaneous tissue of BALB/c mice at 24 h and 96 h; Values are means ± SD using Mann Whitney's *U* test; #: $P < 0.05$ infected *v.s.* PBS treated mice; *: $P < 0.05$ caffeic acid or quercetin-treated *v.s.* untreated mice.

3.8. Caffeic acid and quercetin effect on L. major induced cleavage of p65^{RelA}

The subcutaneous lesions showed positive brown staining to p65^{RelA} antibody, indicating enhanced activation of NF-κB that leads to the cleavage and translocation of the p65^{RelA} subunit to the nucleus. The number of p65^{RelA} positive cells was enhanced by 8.17, and 10.25 fold ($P < 0.01$) at 24 h and 96 h, respectively, compared to PBS-treated mice. Caffeic acid potentiated the density of p65^{RelA} positive cells in pouch membrane (38.5% and 41.0%, $P < 0.05$), at 24 h and 96 h, respectively. Whereas, quercetin treatment inhibited NF-κB by 11% ($P < 0.05$) at 96 h (Figure 10).

4. Discussion

Conventional chemotherapy remains the only curative approach to human leishmaniasis, pending an available vaccine [20,23]. However, the highly toxic side effects and chemo-resistance of chemical drugs requires the development of more efficient medicine. Caffeic acid and quercetin used in this study are major secondary metabolites of many plants used in traditional medicine against leishmaniasis.

Caffeic acid and quercetin are natural antioxidants with low toxicity against mammalian cells [23–26]. Our results showed that they reduce dose-dependently the viability of promastigotes, *in vitro*. Caffeic acid and quercetin induced characteristic features of necrosis and apoptosis. The associated pink or bright blue fluorescence under propidium iodure-Hoechst double labelling was not altered by cell permeable protease inhibitors, thus excluding the involvement of proteases (*i.e.* caspases) in parasite death (Figure 1).

Alternative death pathways resulting in caspases-independent pathways have been reported for *L. major* promastigotes and amastigotes [21,22,32]. Previous *in vitro* studies showed that quercetin inhibited the growth of both promastigotes and amastigotes of *Leishmania donovani* by the blockade of DNA synthesis, while caffeic acid has a strong leishmanicidal activity on *L. major* amastigotes buried in macrophages [24–26]. Apoptosis inducing factor can contribute to caspases independent cell death by affecting mitochondrial function and inducing chromatin degradation [33].

We then used the dorsal air pouch model to mimic the phlebotomine infection in BALB/c mice. *L. major* inoculation drives the massive recruitment of inflammatory cells into the sterile pouch cavity. Interestingly, pouch membranes from *L. major*-stimulated BALB/c mice were thicker and massively invaded by neutrophils within the first hours of infection, followed by macrophages 2–3 days later (Figure 2). This suggests that inflammatory cells joined the membrane, by transmigration of multiple blood microcapillaries (Data not shown). Neutrophils can host viable promastigotes (Figure 5) [16]. Once recruited to infectious sites, neutrophils can form NETs by the extracellular release of mixed nuclear and cytoplasmic contents [6–8]. Different species and strains of *Leishmania* can induce NETs formation and parasite clearance by Th1 polarised neutrophils functions [34]. Alternatively, NETs compete with the early recruitment and activation of dendritic cells, leading to exacerbated cutaneous lesions and persistence of parasite [35].

Some features of phagocytosis mimic NETs formation in caffeic acid-treated BALB/c mice, suggesting that caffeic acid can induce NETs formation, in a ROS dependent way (Figures 2De and 8).

Infected neutrophils persisted 24 h post infection, but superoxide anion production is altered in this cells (Figure 7) [11,36]. Consequently, not all internalized parasites could be killed, as attested by morphologically intact parasites detected in neutrophils of infected mice (Figures 5 and 6). The phagocytosis

of infected apoptotic neutrophils by macrophages represents a silent way for parasites dissemination that could explain the disease promoting effect of neutrophil in *L. major* infection [4,16]. Amastigotes and promastigotes drive down superoxide anion production by inhibiting nicotinamide adenine dinucleotide phosphate oxidase 2 activity (Figure 7) [36,37].

Caffeic acid and quercetin trigger early generated ROS *ex vivo* by infected neutrophils, while caffeic acid alone controls ROS generation by both neutrophils and macrophages at the late phase of infection, leading to rapid apoptosis [38–40]. We showed that quercetin induced cell death by apoptosis and necrosis (Figures 5–7). Recent reports indicated that quercetin can induce a ROS-dependent and caspases-independent necrosis [38,39].

Failure to cure cutaneous leishmaniasis was associated with reduced ability of neutrophils to secrete TNF-α. Moreover, their phagocytosis by macrophages of susceptible BALB/c mice leads to the inhibition of TNF-α production by macrophages of susceptible mice [13]. *L. major* infected neutrophils release TNF-α that inhibits leukocytes migration by a NO dependent mechanism (Figures 8 and 9). Our results showed that infected cells treated with caffeic acid have higher levels of NO, suggesting a direct anti-amastigotes effect of caffeic acid against *L. major* through macrophagic iNOS activation. Radtke *et al.* demonstrated that caffeic acid activates *Leishmania*-infected macrophages for the release of TNF-α, interleukin-6 and interferon gamma. In contrast, there is no significant potential of this polyphenol on TNF-α or interleukin-6 induction in non-infected host cells, attesting for the selective immuno-modulatory effect [25].

TNF-α up-regulation by caffeic acid or quercetin could inhibit phagocytes migration as assessed by the parallel reduction of MPO and ANAE activities. Importantly, apoptotic neutrophils harbouring apoptotic parasites are shown exclusively in caffeic acid or quercetin-treated mice (Figures 5 and 6), suggesting that caffeic acid and quercetin treatment could suppress *L. major* induced apoptosis delay *in vivo*.

Therefore, *L. major* infected macrophages from susceptible (BALB/c) and resistant (C57BL/6) mice can resist to apoptosis [16–18]. However, the mechanism by which *Leishmania* parasites achieve the inhibition of apoptosis is yet unknown.

Recent studies showed that activation of the transcription factor NF-κB protects cells from TNF-α induced apoptosis, and that pharmacological inhibition of ERK1/2 activation reversed the delay of apoptosis induced by *L. major* infection. Whereas, inhibition of p38 MAPK signalling, during *Leishmania* infection had no effect on parasite-induced resistance to apoptosis [41,42].

Infection with *L. major* increased the level of NF-κB $p65^{RelA}$ subunit in agreements with previous study showing that infection of macrophages with *Leishmania* promastigotes *in vitro* causes specific cleavage of the NF-κB $p65^{RelA}$ subunit (Figure 10) [43].

We hypothesize that caffeic acid induced NF-κB expression in infected mice could result from enhanced apoptosis and activation of TNF-α, rather than a direct activation of NF-κB. For Neuzil *et al.* [44], the cleavage of $p65^{RelA}$ is a consequence of caspases activation (Figure 8). The weak control of NF-κB level by quercetin, may be due to the necrosis mechanism of cell death (Figure 9).

In conclusion, restoring apoptosis of infected phagocytes by caffeic acid and quercetin could be related to the anti-leishmanial activity demonstrated on both *L. major* promastigotes and amastigotes, as it could be also associated with antioxidant/pro-oxidant, anti-inflammatory and immuno-modulatory activities of these two major naturally occurring secondary metabolites in plants. Our results associated with previous studies reveal a decisive potential for caffeic acid and quercetin for the development of safer pharmacological drugs against leishmaniasis.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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References

[1] Akhoundi M, Kuhls K, Cannet A, Votypka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. *PLoS Negl Trop Dis* 2016; **10**: e0004349.

[2] Hartley MA, Drexler S, Ronet C, Beverley SM, Fasel N. The immunological, environmental, and phylogenetic perpetrators of metastatic leishmaniasis. *Trends Parasitol* 2014; **30**(8): 412-22.

[3] Harrat Z, Boubidi SC, Pratlong F, Benikhlef R, Selt B, Dedet JP, et al. Description of a dermatropic *Leishmania* close to *L. killicki* (Rioux, Lanotte & Pratlong 1986) in Algeria. *Trans R Soc Trop Med Hyg* 2009; **103**: 716-20.

[4] Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. *Immunobiology* 2008; **213**: 183-91.

[5] Silva MT. Macrophage phagocytosis of neutrophils at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. *J Leukoc Biol* 2011; **89**(5): 675-83.

[6] Remijsen Q, Berghe TV, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res* 2011; **21**(2): 290-304.

[7] Guimarães-Costa AB, Nascimento MTC, Wardini AB, Pinto-da-Silva LH, Saraiva EM. ETosis: a microbicidal mechanism beyond cell death. *J Parasitol Res* 2012; **2012**: 929743.

[8] Rochael NC, Guimarães-Costa AB, Nascimento MT, DeSouza-Vieira TS, Oliveira MP, Garcia e Souza LF, et al. Classical ROS-dependent and early/rapid ROS-independent release of neutrophil extracellular traps triggered by *Leishmania* parasites. *Sci Rep* 2015; **5**: 18302.

[9] Van Assche T, Deschacht M, da Luz RAI, Maes L, Cos P. *Leishmania*-macrophage interactions: insights into the redox biology. *Free Radic Biol Med* 2011; **51**(2): 337-51.

[10] Sansom FM, Tang L, Ralton JE, Saunders EC, Naderer T, McConville MJ. *Leishmania major* methionine sulfoxide reductase a is required for resistance to oxidative stress and efficient replication in macrophages. *PLoS One* 2013; **8**(2): e56064.

[11] Safaiyan S, Bolhassani A, Nylen S, Akuffo H, Rafati S. Contribution of human neutrophils in the development of protective immune response during *in vitro Leishmania major* infection. *Parasite Immunol* 2011; **33**: 609-20.

[12] Mollinedo F, Janssen H, de la Iglesia-Vicente J, Villa-Pulgarin JA, Calafat J. Selective fusion of azurophilic granules with *Leishmania*-containing phagosomes in human neutrophils. *J Biol Chem* 2010; **285**: 34528-36.

[13] Ribeiro-Gomes FL, Sacks D. The influence of early neutrophil-*Leishmania* interactions on the host immune response to infection. *Front Cell Infect Microbiol* 2012; **2**: 59.

[14] Carneiro PP, Conceição J, Macedo M, Magalhães V, Carvalho EM, Bacellar O. The role of nitric oxide and reactive oxygen species in

the killing of *Leishmania braziliensis* by monocytes from patients with cutaneous leishmaniasis. *PLoS One* 2016; **11**(2): e0148084.
[15] Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Müller K, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* 2002; **169**(2): 898-905.
[16] Corral MJ, Benito-Peña E, Jiménez-Antón MD, Cuevas L, Moreno-Bondi MC, Alunda JM. Allicin induces calcium and mitochondrial dysregulation causing necrotic death in *Leishmania*. *PLoS Negl Trop Dis* 2016; **10**(3): e0004525.
[17] Cowburn AS, White JF, Deighton J, Walmsley SR, Chilvers ER. z-VAD-fmk augmentation of TNFα-stimulated neutrophil apoptosis is compound specific and does not involve the generation of reactive oxygen species. *Blood* 2005; **105**(7): 2970-2.
[18] Ruhland A, Leal N, Kima PE. *Leishmania* promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. *Cell Microbiol* 2007; **9**: 84-96.
[19] Gutiérrez-Kobeh L, de Oyarzabal E, Argueta J, Wilkins A, Salaiza N, Fernández E, et al. Inhibition of dendritic cell apoptosis by *Leishmania mexicana* amastigotes. *Parasitol Res* 2013; **112**(4): 1755-62.
[20] De Luca PM, Macedo ABB. Cutaneous leishmaniasis vaccination: a matter of quality. *Front Immunol* 2016; **7**(4): 151.
[21] Gannavaram S, Debrabant A. Programmed cell death in *Leishmania*: biochemical evidence and role in parasite infectivity. *Front Cell Infect Microbiol* 2012; **2**: 95.
[22] Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol* 2005; **6**(3): 268-75.
[23] Alviano DS, Barreto AL, Dias F-de A, Rodrigues Ide A, Rosa Mdo S, Alviano CS, et al. Conventional therapy and promising plant-derived compounds against trypanosomatid parasites. *Front Microbiol* 2012; **3**: 283.
[24] Passero LF, Laurenti MD, Santos-Gomes G, Soares Campos BL, Sartorelli P, Lago JHG. Plants used in traditional medicine: extracts and secondary metabolites exhibiting antileishmanial activity. *Curr Clin Pharmacol* 2014; **9**(3): 187-204.
[25] Radtke OA, Foo LY, Lu Y, Kiderlen AF, Kolodziej H. Evaluation of sage phenolics for their antileishmanial activity and modulatory effects on interleukin-6, interferon and tumour necrosis factor-alpha-release in RAW 264.7 cells. *Z Naturforsch C* 2003; **58**(5–6): 395-400.
[26] Miranda MM, Panis C, da Silva SS, Macri JA, Kawakami NY, Hayashida TH, et al. Kaurenoic acid possesses leishmanicidal activity by triggering a NLRP12/IL-1β/cNOS/NO pathway. *Mediat Inflamm* 2015; **2015**: 392918.
[27] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**(1–2): 55-63.
[28] Verma NK, Dey CS. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. *Antimicrob Agents Chemother* 2004; **48**(8): 3010-5.
[29] Rolas L, Makhezer N, Hadjoudj S, El-Benna J, Djerdjouri B, Elkrief L, et al. Inhibition of mammalian target of rapamycin aggravates the respiratory burst defect of neutrophils from decompensated patients with cirrhosis. *Hepatology* 2013; **57**(3): 1163-71.
[30] Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology* 1984; **87**(6): 1344-50.
[31] Zhou X, Scharf ME, Meinke LJ, Chandler LD, Siegfried BD. Characterization of general esterases from methyl parathion-resistant and -susceptible populations of western corn rootworm (Coleoptera: Chrysomelidae). *J Econ Entomol* 2003; **96**(6): 1855-63.
[32] Casanova M, Gonzalez IJ, Sprissler C, Zalila H, Dacher M, Basmaciyan L, et al. Implication of different domains of the *Leishmania major* metacaspase in cell death and autophagy. *Cell Death Dis* 2015; **6**: e1933.
[33] Isah MB, Ibrahim MA, Mohammed A, Aliyu AB, Masola B, Coetzer TH. A systematic review of pentacyclic triterpenes and their derivatives as chemotherapeutic agents against tropical parasitic diseases. *Parasitology* 2016; **143**(10): 1219-31.
[34] Ricci-Azevedo R, Oliveira AF, Conrado MC, Carvalho FC, Roque-Barreira MC. Neutrophils contribute to the protection conferred by ArtinM against intracellular pathogens: a study on *Leishmania major*. *PLoS Negl Trop Dis* 2016; **10**(4): e0004609.
[35] Hurrell BP, Schuster S, Grün E, Couta M, Williams RA, Held W, et al. Rapid sequestration of *Leishmania mexicana* by neutrophils contributes to the development of chronic lesion. *PLoS Pathog* 2015; **11**(5): e1004929.
[36] Kumar P, Pai K, Pandey HP, Sundar S. NADH-oxidase, NADPH-oxidase and myeloperoxidase activity of visceral leishmaniasis patients. *J Med Microbiol* 2002; **51**(10): 832-6.
[37] Horta MF, Mendes BP, Roma EH, Noronha FSM, Macêdo JP, Oliveira LS, et al. Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. *J Parasitol Res* 2012; **2012**: 203818.
[38] Fonseca-Silva F, Inacio JD, Canto-Cavalheiro MM, Almeida-Amaral EE. Reactive oxygen species production and mitochondrial dysfunction contribute to quercetin induced death in *Leishmania amazonensis*. *PLoS One* 2011; **6**: e14666.
[39] Wang G, Wang JJ, Chen XL, Du SM, Li DS, Pei ZJ, et al. The JAK2/STAT3 and mitochondrial pathways are essential for quercetin nanoliposome-induced C6 glioma cell death. *Cell Death Dis* 2013; **4**: e746.
[40] Passero LF, Bonfim-Melo A, Corbett CE, Laurenti MD, Toyama MH, de Toyama DO, et al. Anti-leishmanial effects of purified compounds from aerial parts of *Baccharis uncinella* C. DC. (Asteraceae). *Parasitol Res* 2011; **108**(3): 529-36.
[41] McCracken JM, Allen LA. Regulation of human neutrophil apoptosis and lifespan in health and disease. *J Cell Death* 2014; **7**: 15-23.
[42] Sarkar A, Aga E, Bussmeyer U, Bhattacharyya A, Möller S, Hellberg L, et al. Infection of neutrophil granulocytes with *Leishmania major* activates ERK 1/2 and modulates multiple apoptotic pathways to inhibit apoptosis. *Med Microbiol Immunol* 2013; **202**(1): 25-35.
[43] Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M. A novel form of NF-kappaB is induced by *Leishmania* infection: involvement in macrophage gene expression. *Eur J Immunol* 2008; **38**(4): 1071-81.
[44] Neuzil J, Schroder A, von Hundelshausen P, Zernecke A, Weber T, Gellert N, et al. Inhibition of inflammatory endothelial responses by a pathway involving caspase activation and p65 cleavage. *Biochemistry* 2001; **40**: 4686-92.