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Immunological Response against *Leishmaniamajor* Parasites with Pyrethrum Extracts

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ABSTRACT

Leishmaniasis is a neglected vector borne disease caused by Leishmania parasites affecting mostly the poor populations of developing countries. Pyrethrum has been shown to have antiviral, antiplasmodial and antitrypanosomal activities, but no reports on antileishmanial activity. Therefore we sought to determine the levels of interferon gamma (IFN-γ) and interleukin 4 (IL-4) cytokines after treatment of infected BALB/c mice with water and methanolic extracts of pyrethrins. IFN-γ and IL-4 cytokines were quantified using ELISA array kit from serum of BALB/c mice. Water and methanol extracts of pyrethrin stimulated production of high IFN-γ and low levels of IL-4 compared to untreated ($P<0.05$). However methanol extracts stimulated more IFN- γ compared to water extracts but not significant ($P=0.4$). Methanol extracts stimulated significantly less IL-4 compared to water extracts ($P=0.032$).Methanol and water extracts stimulated less IFN- γ and IL-4 as compared to pentostam and amphotericin B but not significant ($P>0.05$). This data suggests that water and methanol extracts of pyrethrin stimulates IFN- γ, an inflammatory cytokine against Leishmania parasites. The extracts can be good candidate for further chemothepeutic studies against Leishmania parasites.

Keywords: *Leishmaniasis, cytokines, antileishmanial activity, extracts of pyrethrum, chemotherapeutic;*

INTRODUCTION

The leishmaniases are a group of protozoan diseases caused by the protozoan parasites, *Leishmania* (Kinetoplastida: Trypanosomatidae) transmitted to humans and other mammals by phlebotomine sand flies (Diptera: Psychodidae) [1, 2]. The leishmaniases rank as the leading neglected tropical disease (NTD) in terms of mortality and morbidity with an estimated 50,000 deaths in 2010 [3] and 3.3 million disability adjusted life years [4] .Globally Leishmaniases are endemic in 98 countries, 72 of which are developing nations and 13 correspond to the least developed ones, and considered by the World Health Organization as a Neglected Tropical Disease [2]. Over 350 million people reside in areas with active parasite transmission [1]. Annually, an estimated 1.5–2 million develop symptomatic disease, and approximately 50,000 die, mostly children [2, 5]. The disease is normally divided into three main categories: cutaneous, mucocutaneous, and visceral leishmaniasis. Cutaneous leishmaniasis (CL) is caused by *L. major* and *L. tropica* parasites and usually appears as a self-healing skin ulcer or dermal granuloma that may need several months or years to heal [6]. In some cases, these ulcers can become chronic [7]. While most *Leishmania*species cause lesions confined to small areas of the skin, a few, such as *L. braziliensis,L. aethiopica*cause diffuse lesions that may even spread to mucosal tissues leading to the muco-cutaneous form of the disease [8]. Finally, visceral leishmaniasis, the most severe form of leishmaniasis is caused by *L. donovani* and *L.infantum*. It is characterized by fever, cachexia, hepatosplenomegaly and hyper gamaglobulinemia and, when untreated, can be fatal [9].

In eastern African countries the disease has caused epidemic outbreaks like the ones that occurred in Southern Sudan from 1984–1994 [10], in North-eastern Kenya and South-eastern Ethiopia in 2000–1, in eastern Sudan from 1996–97 [11], in Ethiopia and Eritrea in 1997–

98 [12]. Diffuse cutaneous leishmaniasis (DCL) has been reported in Bungoma district and the Mount Elgon area [13] in Kenya whereas cutaneous is endemic in counties like Baringo, Machakos, Eastern and North eastern parts of the country. Much of VL is concentrated in East Africa [14] yet little has been reported from the endemic parts of Somalia. In West Africa the cutanious leishmaniasis form of the disease is endemic in Burkina farso, Mali, Niger and Senegal [2].

The growing incidence of parasitic resistance against generic pentavalentantimonials, specifically for visceral disease is a serious issue in *Leishmania* control. Notwithstanding the two treatment alternatives, that is amphotericin B and miltefosine are being effectively used but their high cost and therapeutic complications limit their use in endemic areas. In the absence of a vaccine candidate, identification, and characterization of novel drugs and targets is a major requirement of leishmanial research [15].

The cure of infection with *Leishmania* has been demonstrated to beassociated with strong IFN-γ and IL-12 responses in the absence of classical Th2 cytokines(IL-10 and IL-4)[16].Studies have shown that IFN-γ production is associated with healing of *L. major*-infected C57BL/6 mice, while IL-4 production is associated with susceptibility in the BALB/c mice which is evidenced by IFN-γ knockout(KO) mice fail to cure infection [17]. Studies have shown that susceptibility of BALB/c mice to *leishmania* parasites is characterized by early IL-4 synthesis in the absence of IL-12 [18] which has been evidenced in IL-4KO and anti-IL- 4 antibody treated BALB/c mice experiments [19].

Plants have been long recognized as a rich source of biologically active extracts, essential oils, and isolated substances. In fact, research laboratories around the world search in plants for active substances against diverse illnesses such as microbial and protozoal infections, cancer, diabetes, and inflammatory processes e.g *Warbugia ugadensis* has been shown to stimulate production of IFN-γ and low levels of IL-4 in *Leishmania major* infected BALB/c mice[20, 21]. Plant-derived natural products such as phenolic compounds, steroids, quinones, coumarins, terpenoids, and alkaloids have been widely investigated for their anti-leishmanial potential [22, 23].

This study aimed at investigating the immunostimulatory potential of water and methanol extracts of pyrethrum against *L. major* infected BALB/c mice.

Materials and Methods

Study Design

This was a laboratory experimental study. The study was carried out in *Leishmania* laboratory at Centre for Biotechnology Research and Development, Kenya Medical

Research Institute. Pyrethrum plant was collected from Baragoi, West Pokot County and transported to CTMDR for extraction of pyrethrin.

In Vitro L. Major parasite Culture

Leishmaniamajor parasitestrain National *Leishmania* Bank (NLB)-144 was cultivated from a frozen stabilate to stationary phase at 25°C in Schneider's *Drosophila* medium supplemented with heat-inactivated foetal bovine serum, 100 U/ml penicillin, 500 µg/ml streptomycin and 250 µg/ml 5-fluorocytosine arabinoside. The parasites were harvested, centrifugally washed at 1500 revolutions per minute for 15 minutes at 4°C. These were then used for both *in vitro* and *in vivo* tests. For infection of mice foot pads, $1x10^6$ promastigotesin 40 µl phosphate-buffered saline were used.

Extraction of Pyrethrin

Pyrethrins were extracted from pyrethrum flowers as described by shawkat *et al.*, [24]. Briefly the flowers were extracted with petroleum ether at room temperature (3 x 24 hours) and filtered. The filtrate was further partitioned with water and methanol at a ratio of 4:1 (filtrate: methanol) vigorously shaken and allowed to settle. Yellowish layer containing pyrethrins was carefully removed and dried in a vacum using a rotary evaporator.

We quantified pyrethrinsusinga method described by [25] with some modification was adopted. Briefly, extracted pyrethrins were dissolved in acetonitrile and filtered using a 0.22 µm filter. They were analyzed using a Beckman Coulter system Gold 126P solvent module HPLC. Separations was done on a C18 column (150 x 3.9 mm) using a solvent mixture of acetonitrile and water at a flow rate of 1.4 ml/minute. Absorbance was monitored at a wavelength of 225 nm. Pyrethrins in the extract were quantified on the basis of their retention time in comparison to the reference standard. Since individual pyrethrins were not available as pure compounds, quantification method was used, a commercial

pyrethrum mixture with an estimated amount of 25% total pyrethrins as the reference solution. This was obtained from the Pyrethrum Board of Kenya. The amount of total pyrethrins in the assayed sample was estimated by calculating the sum of measured peak areas of individual pyrethrins [25].Ten mg of Pyrethrins were reconstituted by dissolving in 10ml of PBS solution to obtain the stock solution of 1mg/ml.

Infection of Mice, Treatment and Serum Collection

Female BALB/c mice were infected in the hind footpad with 1 × 10^6*L. major* meta cyclic promastigotes (10µl). In all experiments, treatment was initiated 4 weeks after infection had established as determined by the presence of lesions. The extracts were administered both orally (using a cannula) and intraperitoneally (using 26 gauge needle) daily for 30 days. BALB/c mice were treated with 100ul of 13.6µg/ml water extracts and 8.3 µg/ml methanolic extracts. The untreated group received PBS. The control group was given intramuscularly 20mg/kg body weight daily of Pentostam® and amphotericin B for 4 weeks. Blood was collected from the BALB/c mice tail into 2ml tube. Blood was allowed to clot for 30 minutes at 25°C and centrifuged at 2,000 x g for 15 minutes at 4°C. Top yellow serum layer was pipetted off without disturbing the white buffy layer. Serum was diluted 1:5 with diluted Assay Buffer before assaying.

Serum Interferon-gamma and IL-4 Immunoassay

Serum interferon-gamma immunoactivity was determined using a commercial single analyte ELIS Array kit (SA Biosciences) exactly according to the manufacturer's instructions. Outcomes were quantified by optical density at 450 nm, with background correction at 570 nm, using ELX 800, a universal micro plate reader. The detection levels of interferon-γ and IL-4 was 39pg/ml and 32.8 pg/ml respectively.

Ethical and Bio Safety Considerations

Approval for the study was sought from Kenya Medical Research Institute (KEMRI)Scientific ethical research unit (SERU). The experiments were done in compliance with KEMRI's Animal Care and Use Committee (ACUC).

Statistical Analysis

Data were recorded in Microsoft Excel and imported into SPSS 13.0 for analysis. All experiments were carried out in triplicate. The mean and standard deviation of at least three experiments were determined. Statistical analysis of the differences between mean values obtained for the experimental groups were done by Student's t test. P values of ≤0.05 or less were considered significant.

Results

Serum Levels of IFN-γ and IL-4 Cytokines in *L. Major* Infected BALB/c Mice Treated with Water, Methanol Extracts and PBS

Generally it was observed that IFN-γ was higher than IL-4 in water and methanol extracts treated BALB/c mice (Figure 1)

BALB/c mice treated orally with methanolic extracts of pyrethrin stimulated more production of IFN gamma levels than those treated with water extracts but the difference was not significantly different (Mean= 11.5±42.31, t=0.2718, P=0.40). It was observed that both methanol and water extracts stimulated significantly high production of IFN-γ than PBS treated mice. Water and methanol extracts treated BALB/c mice had significantly low levels of IL-4 cytokines as compared with PBS treated mice (Figure 1).

It was observed that methanol extracts of pyrethrum administered intraperitoneally stimulates high production of IFN-γ than water extracts but not significant and vice versa for IL-4 production. BALB/c mice treated intraperitoneally with pentostam stimulated more production of IFN gamma than the methanolic extracts of pyrethrin treated mice but the difference was not significant.(P=0.2088), however methanolic extracts of pyrethrin stimulated less amounts of IL-4 compared to pentostam treated mice and the difference was not significant (P=0.1178).

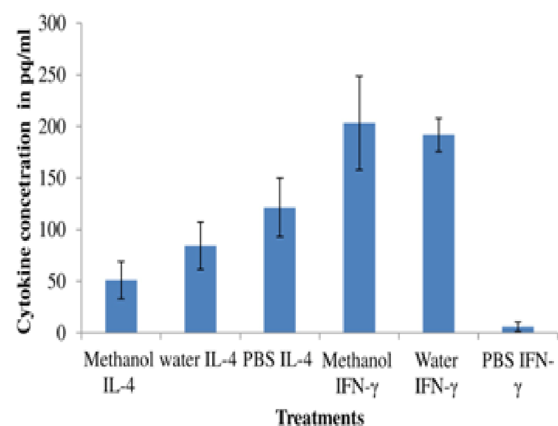

Figure1. *IFN-γ and IL-4 cytokine levels after oral treatment*

This study also found out that BALB/c mice treated intraperitoneally with amphotericin B stimulated more production of IFN gamma than the methanolic extracts of pyrethrin treated mice but the mean difference was not significant (P=0.3818).BALB/c mice treated intraperitoneally with methanolic extracts of pyrethrin stimulated significantly less amounts of IL-4 production than the amphotericin B treated mice (P=0.05). BALB/c mice treated intraperitoneally with methanolic extracts of pyrethrin significantly stimulated more production of IFN gamma than the PBS treated mice (P=0.05), however methanolic extracts of pyrethrin stimulated significantly less amounts of IL-4 than PBS treated mice (P=0.03).

BALB/c mice treated intraperitoneally with water extracts of pyrethrin stimulated less IFN gamma and IL-4 production than the pentostam treated mice but the difference was not significant. BALB/c mice treated intraperitoneally with water extracts of pyrethrin significantly stimulated less of IFN gamma production than the amphotericin B treated mice (P=0.022), however water extracts stimulated less amounts of IL-4 than the amphotericin B treated mice but the difference was not significantly different (P=0.3055)

BALB/c mice treated intraperitoneally with water extracts of pyrethrin significantly stimulated high amount of IFN gamma production than the PBS (-ve control) treated mice (P=0.0141). BALB/c mice treated intraperitoneally with water extracts of pyrethrin significantly stimulated less amounts of IL-4 than the PBS treated mice (P=0.0445) (Figure 2).

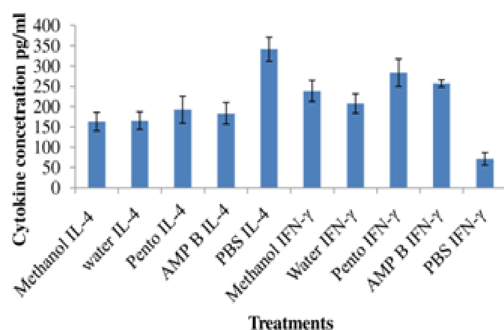

Figure2. *IFN-γ and IL-4 cytokine levels after IP treatment*

Discussion

Traditional herbal medicine is gaining increased attention as they can reduce the risk of chronic diseases and act as antibiotics, antioxidants, and/or immunomodulators. Several studies have described the effects of plant extracts or isolated compounds in immune cells and cytokine production [26].

Thus, the study of active compounds obtained from plants used in traditional medicine plays a pivotal role in the search for new antileishmanial molecules [27, 28].

Several raw extracts from different plants have been shown to exhibit antileishmanial activity, which may not only be due to their direct action on the parasite, but also due to a concomitant effect on the host immune response [28].

Natural products such as alkaloids, terpenes,quinones, and polyphenols have shown potent growth inhibition of *T. cruzi* and *Leishmaniabrasiliensis* [29].Pyrethrum plant is rich in secondary metabolites like alkaloids, gallic, cathechic, tannins, flavonoids and triterpenes which have shown antibacterial activity [30]

Methanolic and water extracts of *C. cinerariefolium* on *L. major* infected BALB/c mice exhibited decreased lesion sizes and parasite burden in the spleen. In our study this may be attributed to exhibited high amounts of IFN-γ production by CD4+T cells and low levels of IL-4 production associated with susceptibility in the BALB/c mice [31].

Conclusion

Water and methanol extracts of *C. cinerariefolium* stimulated T cells to produce high levels of IFN-γ and low IL-4 cytokines in *Leishmania major* infected BALB/c mice and from these results more studies are recommended on pyrethrum extract against *Leishmaniasis*.

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