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In vitro Anti *Mycobacterium tuberculosis* H37Rv Activity of *Lannea acida* A. Rich from Burkina Faso

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Abstract: The cytotoxic and anti-Mycobacterium tuberculosis H37Rv activities of hydro-alcoholic extract of *Lannea acida* A. Rich (Anacardiaceae) were assessed. The cytoxicity evaluation was carried out on THP1 monocytoid cell line (after 24 h at 1; 5 and 10 μg mL^{-1}) and showed only a slight modification of lactate dehydrogenase (LDH) release. The rate of monocytes in different stages of mitosis had been amended in absence and presence of extract as follows: Go/ G1 58.83 - 59.83%; synthesis 21.95 - 18.64%; mitosis 16.67 - 15.77%; necrosis 2.65 - 5.64 %. The percentage of inhibition of Mycobacterium tuberculosis proliferation was respectively 77.6 and 36.8% at 1.2 and 0.6 mg mL^{-1} of extract. This is an interesting experimental study on antimicrobial and immune-stimulating properties of *Lannea acida* ethanol-water (70%v/v) extract which may contain potential antibacterial and immune-stimulating agents for clinical use.

Key words: *Lannea acida* barks, anacardiaceae, ethanolic extract, cytotoxicity, anti *Mycobacterium tuberculosis* H37Rv

INTRODUCTION

Tuberculosis is a systemic disease caused by *Mycobacterium tuberculosis*. In the recent years, tuberculosis is a really well-known public health problem with more than 90% of cases in developing countries, particularly in sub-Saharan Africa. Approximately eight millions of new cases occur annually with 2 million deaths (Dye *et al*., 1999; WHO, 2004). This high prevalence could be due to several factors including, promiscuity, malnutrition, illiteracy, inadequate medical infrastructure, HIV infection (Berenguer *et al*., 1992) and the ineffectiveness of currently used clinical agents synthetic chemicals against multi-resistant strains.

In spite of our current knowledge, African countries still enclose a biodiversity of plants not yet explored or not rationally exploited (Gaillard *et al*., 2006). Today, interest in medicinal plants is increasingly growing due to their antimicrobial and pharmacological properties. *Lannea acida* is a perennial tree about 8 to 12 m height with scaly bark, blackish-tranche red and yellow striped fiber and is widely distributed in the Sudanian and Guinean savanna from Senegal to Central African Republic (Arbonnier, 2002; Mahamane et *al*., 2007). It is traditionally used (combined or sole) against conjunctivitis, dysentery and sores (Arbonnier, 2002). In the northern Ivory Coast, it is used in treatment of diarrhoea, stomach aches, gonorrhoea and rheumatism (Kone *et al*., 2004). *Lennea acid*a (bark, leaves and roots) is generally used as decoction, infusion or maceration against fever, malaria, diarrhoea, skin diseases, coughs and dysentery (our own investigations in western Burkina Faso, June, 2005).

A few previous studies describe pharmacological properties of *L. acida* in comparison with other plants of the same genus such as *L. kerstingii, L. nigritana* or *L. microcarpa*. Thus *L. acida* has not been investigated for its chemical composition, only a preliminary screening revealed the presence of alkaloids and tannins in the bark (Etuk *et al*., 2009). The few studies conducted on the pharmacological properties of the plant are about its antidiarrhoeal properties. The results reveal that the bark

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of the plant was active against several pathogenic bacteria including *Vibrio cholerae* (Kone *et al*., 2004; Etuk *et al*., 2009; Akinsinde and Olukoya, 1995).

On the other hand there is few scientific data on the antibacterial and immune-stimulating properties of the plant. According to our literature survey, anti mycobacterial properties of the plant have not previously been published. The present study is carried out in the aim to investigate the effect of *Lannea acida* bark ethanol extract on the monocytes THP-1 and anti-*Mycobacterium tuberculosis* $H37R_v$ activities before and after human macrophages infection.

MATERIALS AND METHODS

Chemicals: Dimethylsulfoxide (DMSO) and Tween-80 were purchased from Merck (Germany), anti--CD14 magnetic beads (Miltenyi Biotec Bergish Gladsbach, Germany), RPMI 1640, L-glutamine and Gentamicine were purchased from Gibco BRL, FCS (BioWhittaker, Belgium), Middlebrook 7H10 (Becton Dickinson, MD) and saponin (Sigma, St Louis, MO,USA) respectively.

Plant material: *L. acida* (Anacardiaceae) barks were harvested in July 2005 from Noumoudara, 25 km Southern Bobo-Dioulasso, Burkina Faso. Voucher specimens were identified by Pr Millogo and deposited at the department of Plant Biology and Ecology, University of Ouagadougou. The barks of *L. acida* were dried at room temperature.

The extraction was performed by cold maceration in a mixture of ethanol-water (70% v/v). One hundred gram of dry powder of *L.acida* was percolated with 500 mL of the mixture ethanol-water (70%, v/v) for 48 h. The extract was filtered and concentrated to dryness under reduce pressure and then was lyophilized. Ethanol was removed under reduced pressure in a rotary evaporator (BÜCHI Rotavapor R-200, Switzerland) and the residual aqueous solution lyophilized using a freeze drying system (Lyovac GT2).The crude extract was stored at 4°C for further use.

Evaluation of *L. acida* crude extract stimulation on cell viability and cell cycle: THP1 monocytes (10^6 cells per well) and 100 μL of extract at different concentrations (1 to 100 μg mL^{-1}) were put in culture, in triplicate. The supernatant was collected and frozen after 4 h, 2, 3 and 6 days from stimulation. Cytotoxicity was evaluated by measuring the presence of lactate dehydrogenase (LDH) in the cell culture supernatant by "CytoTox96 Kit" (Promega, Madison, WI, USA), used according to manufacturer's instruction. The cell cycle represents the total period of division, that is to say all the morphological and biochemical events that are responsible for cell proliferation. The flow cytometry provides a quick and easy method for the analysis of the cell cycle. It allows monitoring the distribution of cells in different phases of the cycle according to different stimuli or the addition of certain drugs. In this study, this method has been used to evaluate the impact of the ethanol extract of *L. acida* (bark) on the different phases of the cycle of THP-1 monocytes. Briefly, monocytes (10^6 cells mL^{-1}) were cultured in the presence of ethanol-water extract of *L.acida* (final concentration is 10 μg mL^{-1}) in a culture plate. After 72 h 400 μL propidium iodide solution, consisting of 50 μg mL^{-1} propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100 (Sigma) was added to the culture. After incubation at 4°C for 45 min, we determined the proportion of cells at different stages of cell division by flow cytometry (Becton Dickinson, FACS Calibur).

Anti-*Mycobacterium tuberculosis* assay: This experiment consisted of three parts: Separation of monocytes and macrophages from blood from healthy donors by density gradient centrifugation and continuous culture and stimulation (with extract) of macrophages before their infection and after infection with *Mycobacterium tuberculosis* H37Rv, Cultivation of microorganisms (*Mycobacterium tuberculosis* H37Rv) from the lysis of macrophages on middle 7H 10 for colony forming unit (CFU) count.

Separation of monocytes and macrophages from whole blood: Briefly the technique can be described as follows: The blood of healthy individuals collected in heparin anticoagulant was centrifuged for 20 min at 1800 rpm. All lymphocytes, monocytes and granulocytes were collected and placed in 50 mL PBS (1%) or complete RPMI(Roswell Park Memorial Institute Medium)(RPMI with 10% serum). The pellet was resuspended, after three lavages in 10-15 mL of PBS and a positive separation was done using magnetic beads conjugated with anti-CD 14.

Separation of $CD14^+$ monocytes: Marking of monocytes with magnetic beads anti-$CD14^+$: In details 250.10^6 cells were resuspended in 100 μL PBS and added to 100 μL of beads with specific antibodies to $CD14^+$ and incubated at 4°C for 20 min. The column on the magnetic media is wet with 5 mL of PBS. The total cell suspension was gently introduced into the column. The $CD14^+$ monocytes are retained by the column and cells were recovered in a tube below the column. The $CD14^+$ monocytes attached to the column were washed successively 3 times with 5 mL of PBS. These $CD14^+$ monocytes were counted under an

optical microscope using counting chamber (10^6 = number of cells accepted by tile = 30×10^6).

Separation of macrophages by adherence: All $CD14^+$ monocytes were cultured in RPMI complete (5×10^6 cells to 8 mL of complete RPMI). After 2-3 h of adherence the non-attached cells were washed and 10 mL of complete RPMI was added to the flask.

Continuous culture of monocyte-macrophage $CD14^+$ and infection by *Mycobaterium tuberculosis*: The strain of *Mycobacterium tuberculosis* H37Rv was kindly provided by the laboratory of Biology and immuno-pathology of Tor Vergata University (Roma, Italy). *M. tuberculosis* H37Rv strain was transferred every 2 months and the growth was performed in Sauton medium as a monolayer previously described by Schmitz *et al.* (1993). In order to infect macrophages, bacilli layers were collected and sonicated to enhance a homogeneous suspension. A CFU count was performed in order to identify the titre before storage of the aliquots at -80°C.

Representative samples were thawed and the enumeration of colonies (CFU mL^{-1}) was performed on Middlebrook 7H10 (Becton Dickinson, MD) supplemented with OADC (oleic acid-albumin-dextrose-catalase). Inocula prepared from suspension of mycobacteria in RPMI medium were sonicated for 2 min and used to infect human MDMs. Macrophages were infected at a Multiplicity of Infection Monocytes/MTB of 1:10 by adopting the technique of micro dilution plate (24 wells) using 1.5×10^6 macrophages and 15×10^6 *M. tuberculosis* for each well.

The stimulation with *L. acida* ethanol-water extract (0.6 and 1.2 mg mL^{-1}) was performed according to two scenarios: First, at the same time as infection and a second time, 4 h after infection. The infection lasted 4 h in both the stimulation conditions. At the end of 4 h, the supernatants from each well were discarded Each well was completed in triplicate. Two different concentrations of the extract (0.6 and 1.2 mg mL^{-1}) were compared (Fig. 1).

After 7 days of culture,supernatants were recovered and the macrophages were lysed with 0.1% saponin in PBS / Tween 100 for release and count of *M. tuberculosis* H37Rv (Merck, Germany). Aliquots of each tube containing the cells of *Mycobacterium tuberculosis*, at dilutions of 1/10, 1/100, 1/1000 and 1/10000 were transplanted on 7 H10 medium in petri dishes in triplicate. Cultures were incubated at 37°C for 3 weeks and CFUs were determined.

Determination of colony forming units (CFUs): The bacilli in the culture supernatants and those released after cellular lyse were placed in plates after 3 h incubation with cells to determine the percentage of phagocytosis at 1,3,7 and 11 days.

Intracellular bacteria were obtained by lysing cells with sterile PBS containing 0.1% saponin and released bacteria were serially diluted in PBS supplemented with 0.01% Tween-80 (Merck, Germany). Finally, the bacteria were inoculated in the medium 7H10 with OADC in triplicate. The CFU was determined after 14 days of incubation at 37°C. The colonies were maintained in this condition for 30 days to prevent the occurrence of other colony.

Statistical analysis: Each value is the mean (±SD) of inhibition percentage. The standard deviations for each mean value were calculated with Microsoft Excel 2007.

RESULTS AND DISCUSSION

Effect of crude extract on cell viability and cell cycle: In order to assess the possible toxic effect on THP1 cells of the different dilutions of the extract, cytotoxicity was monitored in terms of LDH released in the supernatant of stimulated and unstimulated cells (Fig. 1, panel A and B). The proportion of LDH in the extracellular medium is proportional to the degree of toxicity (Matthew and Rushton, 1994).

Particularly, data of the Fig. 1a (Panel A: from 1 to 10 μg mL^{-1}) shows a low level of toxicity during the first three time points at 144 h; the absorbances of the controls are slightly higher than those of well-tests. At 144 h of cultivation, the absorbance at 1 μg mL^{-1} (absorbance = 3.53±0.92) is slightly higher than the control without extract (absorbance = 3.42±0.79). Moreover, data in Fig. 1 (panel B: from 20 to 100 μg mL^{-1}) show that all absorbances of the controls are slightly higher than those of well-tests.

The impact of crude extract was also assessed on cell cycle and evaluated by flow cytometry in terms of propidium iodide incorporation. Figure 2 showed a very slight modification in the percentage of monocytes at the different stages of cell cycle following stimulation with 10 μg mL^{-1} of crude extract.

Anti-*Mycobacterium tuberculosis* activity: When macrophages were stimulated with the extract prior to infection with *M. tuberculosis*, the percentages of inhibition were 8.8 and 7.36%, respectively at 1.2 and 0.6 mg mL^{-1} with no significant difference at the 5%. On the other hand, when macrophages were infected

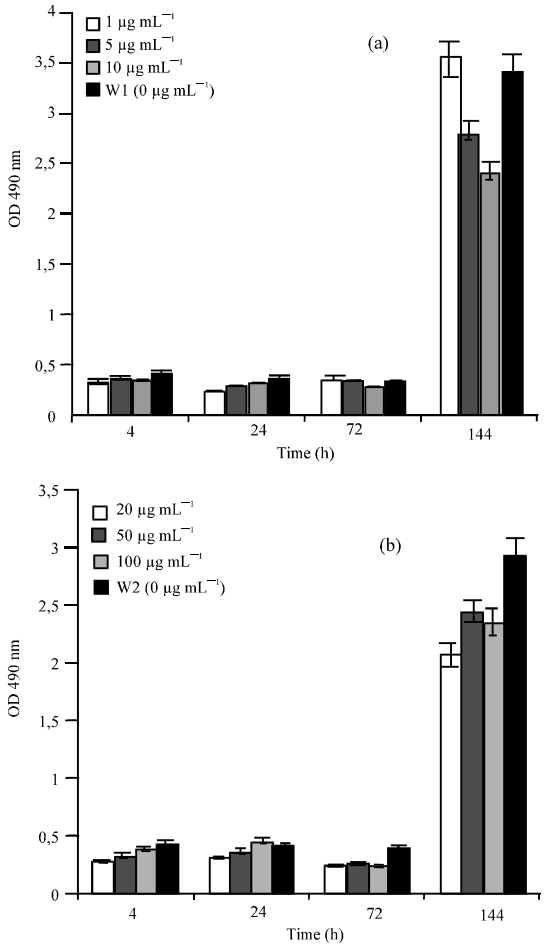

Fig. 1: Cell viability following stimulation with crude extract from *L. acida*. Briefly, THP1 cells were stimulated with 1, 5, 10 µg mL^{-1} (panel A) or 20, 50, 100 µg mL^{-1} (panel B) of the *L. acida* crude extract and monitored for LDH release at 4 h, 1 day, 3 days and 6 days after stimulation. (a) Panel A (from 1 to 10 µg mL^{-1}) and (b) Panel B (from 20 to 100 µg mL^{-1}) NB: OD is Optical Density or Absorbance

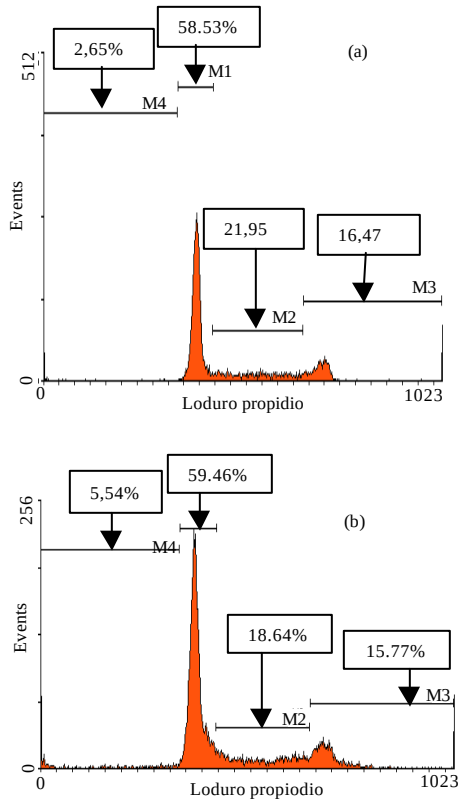

Fig. 2: Cell cycle representation of monocytes following stimulation with *L. acida* crude extract.

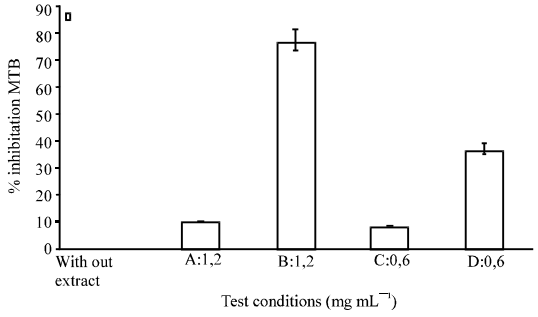

Fig. 3: Effect of *L.acida* A.Rich ethanol extract on *Mycobacterium tuberculosis* H37Rv (MTB) in Macrophage

with *Mycobacterium tuberculosis* prior to the extract stimulation, the percentages of inhibition were 77.6 and 36.8%, respectively at 1.2 and 0.6 mg L^{-1} with significant differences at 5%. The inhibitory activity of the extract in the first case (stimulation before infection) is lower than in the second case (infection before stimulation) with significant differences at 5% (Fig. 3).

The present study aimed to evaluate the anti mycobacterial activity of *L. acida* bark ethanol-water extract and to evaluate the toxicity of the extract on Monocytes THP 1. The plant was selected after inquiries is the most frequently used part in the traditional medicine and this is often used as decoction or the powder is dissolved in alcohol and administrated by oral route. Hence the extract in the present study was prepared according to the traditional use.

The following stages are recorded with the following cell percentages. M1 indicates cells in G0/G1 phase; M2 indcates cells in synthesis phase; M3 indicates cells in G2/mitosis phase; M4 indicates cells in necrosis. Briefly, cells were stimulated (Fig. 2b) or not (Fig. 2a) with 10 μg mL^{-1} of crude extract for 72 h and monitored for propidium iodide incorporation by flow cytometry.

The results revealed that, at 1; 5 and 10 μg mL^{-1} the crude extract exhibited no significant toxicity against monocytes THP 1. After 4, 24, 72 and 144 h and at 20, 50 and 100 μg mL^{-1}, the quantity of external LDH was slightly lower than that of the control. In this case, the extract showed no toxicity between 1 to 100 μg mL^{-1} on monocytes THP 1. We could even say that the extract had a slight stimulation of monocytes, with significant differences at the 5%. This hypothesis was confirmed by the effect of the extract on the cellular cycle of monocytes THP 1. In the absence of extract, the percentages of cells in different phases of cellular division were: M1: 58.53%, M2: 21.95%, M3: 16.47% and M4: 2. 65%; while, in the presence of extract at a final concentration of 10 μg mL^{-1}, the distribution during cellular division displayed: M1: 59.46%, M2: 18.64%, M3: 15. 77% and M4: 5.54%. A slight change was observed in the percentages of cells in different phases of mitosis with no statistically significant difference at 5% significance. The slight toxic effect observed with the extract is in accordance with the report of Sowemimo *et al*. (2009) who demonstrated that the plant was fairly toxic on cervix adeno carcinoma cell line.

The anti-mycobacterial assay in the present study showed that, when macrophages were stimulated with the extract before infection with *Mycobacterium tuberculosis*, the percentages of inhibition were 8.8 and 7.36%, respectively at 1.2 and 0.6 mg mL^{-1} with no significant differences at the 5%. However, when macrophages were infected with *Mycobacterium tuberculosis* before stimulation, the percentages of inhibition were 77.6 and 36.8%, respectively at 1.2 and 0.6 mg mL^{-1} with statistically significant differences. The inhibitory activity of the extract in the first case (stimulation before infection) is lower than that in the second case (infection before stimulation) with significant differences at the 5%. These results showed the anti-mycobacterial effects of the bark extract. *Lannea acida* itself has not been well studied for its pharmacological properties; in comparison with the other plants of the same genus, such as *L. kerstingii, L. nigritana* or *L. microcarp*a thus the plant has not been investigated for its chemical composition; only a preliminary screening revealed the presence of alkaloids and tannins in the bark (Etuk *et al*., 2009). The few studies conducted on the pharmacological properties of the plant were about its antidiarrhoeal properties, which resultsrevealed that the bark of the plant was active against several patho genic bacteria including *Vibrio cholerae* (Akinsinde and Olukoya, 1995; Kone *et al*., 2004; Etuk *et al*., 2009). Therefore, it is a challenge to investigate the plant for several pharmacological properties since the plant is of wide usage in traditional medicine.

This study indicates that the hydro-alcoholic extract of *L. acida* A. Rich has no significant toxicity on monocytes THP1.The difference between the percentages of monocytes necrosis in the absence and the presence of extract might be due to the mass effect. There is a slight stimulation of monocytes THP1 which is manifested in the quantity of external LDH; the quantity of external LDH of the control has been higher than those of well-tests. Moreover the crude extract exhibits an anti-*Mycobacterium tuberculosis* activity (77 and 36% respectively to 1.2 and 0.6 mg L^{-1} with significant difference at 5%) when macrophages have been infected before the stimulation with the extract. But, when macrophages were stimulated with the extract before infection, the percentages of inhibition were 8.8 and 7.36, respectively at 1.2 and 0.6 mg L^{-1} with no significant differences at 5%.

CONCLUSION

These results suggest that *L. acida* A. Rich bark could be a new lead establishment of new anti-*mycobacterium* agents for clinical trials. Furthermore this work will be extended with evaluation of the anti-*Mycobacterium tuberculosis* property with different fractions obtained from the crude extract in the aim to isolate the active components and to expand this study to other *Mycobacterium* species.

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