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IN VITRO EVALUATION OF THE ACTIVITY OF AQUEOUS EXTRACT OF A PLANT CODED PHYLLAM (*PHYLLANTHUS AMARUS*) ON TWO STRAINS *OF MYCOBACTERUM TUBERCULOSIS* AND ONE STRAIN OF *MYCOBACTERUM ULCERANS* .

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ABSTRACT

Burili ulcer and tuberculosis are diseases caused by mycobacteria and are a real public health problem in Côte d'Ivoire. We evaluated the activity of the aqueous extract of *Phyllanthus amarus* on the *in vitro* growth of two strains of *M.tuberculosis* (H37Rv and RF94) and one strain of *M. ulcerans* recorded under the name (02003). The results showed that all strains tested were susceptible to the aqueous extract of *Phyllanthus amarus* with minimum inhibitory concentrations (MIC) of 16 mg / ml for RF94 and 64 mg / ml for H37Rv and 02003 strains. This study shows that it is necessary to widen our research on more extracts tested in vitro and in vivo to enhance the bactericidal activity of *Phyllanthus amarus.*

Keywords : *Phyllanthus amarus* , M. *ulcerans* , M. *tuberculosis*, growth *in vitro*.

INTRODUCTION

In tropical countries with low income, mycrobacterioses of the species *M. tuberculosis* and *M. ulcerans* set themselves up as an obstacle to development despite the advancement being made in the health care sector [1, 2]**.** After decades of struggle, the current problem of tuberculosis seems to have definitely slipped into the thorny issue of drug resistance. The emergence of resistant strains of TB can ultimately compromise the control of tuberculosis especially in developing countries [3].

As for Buruli ulcer, after the discovery of the first case in 1978 in the region of Daloa in Cote d'Ivoire endemic after more than twenty years extends to the majority of health districts [4, 5, 6]. According to WHO, Africa bears 99% of the global burden of this disease. 15 endemic countries confirmed in 2012, were mostly countries in West Africa and Central Africa [7, 8]**.** In 2010, the cumulative number of cases of Burili ulcer was estimated to 70,000 cases . The three major endemic countries are Ghana, Benin and Cote d'Ivoire. [7]. In Côte d'Ivoire, this disease remains a public health problem; where we record more than half of the total cases in the world. 48% of affected subjects are children under age 15 living in tropical rural communities characterized by a warm and humid climate near water courses [9]. WHO recommends for the treatment of Burili ulcer the association of antibiotics as firs-line treatment and surgery as second-line treatment. But we noted that in both cases, there are relapses [10].

Traditional medicine is used by the population for curative and preventive purposes [11, 12]. Plants are used in the treatment of many diseases (digestive disorders, oral, lung, liver and skin) [11, 13]**.**

In order to contribute to the search for new antimycobacteriennes molecules , the activities of the aqueous extract of a plant (*Phyllanthus amarus*) coded PHYLLAM, were evaluated *in vitro* on two reference strains of *M. tuberculosis* (H37Rv and RF94) and a strain of *M. ulcerans* registered under the name (02003).

MATERIALS AND METHODS

MATERIALS

Plant material

The plant material consists of leaves of PHYLLAM (*Phyllanthus amarus*) obtained from the Central West region of Côte d'Ivoire. These leaves were dried under shade for two weeks.

The total aqueous extract was prepared from the powder obtained after grinding.

Biological material

The strain of *M. ulcerans* registered under the name (02003) of human origin and two reference strains of *M. tuberculosis* (H37Rv and RF94) provided by the Institut Pasteur of Côte d'Ivoire (IPCI) were used .

Culture medium

The Lowenstein-Jensen (LJ) medium was provided by AES Laboratory. This is the selective medium for the cultivation of mycobacteria whose composition for 600 ml of water is as follows: Phosphate-monophase (2,5g), Magnesium sulphate (0,24g), Sodium Citrate (0,60g), asparagines L (3,60g) potato flour (30g), malachite Green (0,40g). The resulting solution has a pH of 7,1 at 25°C.

METHODS

Preparation of the plant aqueous extract

PHYLLAM leaves were washed and dried under shade and transform into fine powder by using an IKA MAG grinder. The total extract was prepared according to the method developed by **GUEDE- GUINA et al 1993** [14].

100 g of powdered PHYLLAM are dropped into 2 liters of distilled water and then homogenized under magnetic stirring for 24 hours at 80°C using a magnetic stirrer IKA – MAG RCT. The homogenate is filtered twice in succession on cotton wool and then on Whatman 3mm paper. The filtrate is evaporated using a Buchi rotary evaporator 461 Water Bath. The resulting slurry was lyophilized and the resulting powder is the total extract [15].

Determination of antimicrobacteria activity

Dilution Range in ratio of 2

20 g of total aqueous extract are dissolved in 10 ml of sterilized distilled water. A solution M of 2000 mg / ml is obtained. 10 ml of solution M were homogenized in 5.625 ml of distilled water. A first intermediate solution I of 1280 mg / ml concentration was obtained. Two Fold dilutions of solution I are made until the ninth tube containing only distilled water. For sterilization, these nine tubes are autoclaved at 121° C for 15 min.

Incorporation of the total extract into culture medium

To obtain the final solutions, 9 sets of 10 tubes numbered from 1 to 8 with two control tubes. For each of the tubes, 0.5 ml of each solution I was homogenized in 4.5 ml of culture medium. For control tubes (T), the solution I contains only distilled water. The set of tubes is coagulated at 85 ° C for 45 min.

Preparation of the microbial suspension

Some colonies of young cultures of *M. tuberculosis* and *M. ulcerans* are removed and suspended in distilled water to obtain a standard comparable to BCG mycobacteria corresponding to 10^6 ml water opacity. This tube is considered a dilution tube of 10^0. 0.5 ml of the suspension 10^0, is homogenized in 4.5 ml of distilled water to obtain suspension 10^{-1}. We proceeded in the same manner for the suspension 10^{-3}, 10^{-4}, 10^{-5}.

For each strain, we inoculated 10 tubes at a dilution of 10^{-3} at 0.2 ml per tube. All the 10 tubes in each case composed of two control tubes concentration of 0 mg / ml of plant extract and eight tubes containing plant extract concentration ranging from 1 to 128 mg / ml. The tubes were incubated at 37 ° C for *M. tuberculosis* and 30 ° C for *M. ulcerans* for a period of 8 weeks.

Colony counting

After 8 weeks of incubation, colonies of *M. tuberculosis* and *M. ulcerans*, were counted by direct counting. Mycobacterial growth in eight experimental tubes was measured as a percentage of survival, calculated relative to 100 % survival in the control tubes.

The method of calculating the percentage of survival of mycobacteria in experimental tubes can be summarized by the following formula:

$$S = \frac{n}{N \times 100}$$

S: survival of germs (%)

N: number of colonies in the control tubes

n: number of colonies in the test tube [14, 16].

The MIC is determined according to the method described by **Heifets et al 1988** [17]. This is the lowest concentration of plant extract inhibiting the growth of bacteria that is 1% surviving bacteria compared to that of control tubes. The IC_{50} was determined graphically from the sensitivity curve as a function of plant extract concentrations.

Statistical Analysis

The exploitation of the results was performed using analysis of variables (ANOVA -one way) using the Student "t" test. The difference is considered significant for a probability level of p lower than 0.005. Curves were plotted using the Graph Pad (version 5.I , Software, USA) software.

RESULTS

These results are obtained with a dilution of 10^{-3} after 8 weeks of incubation at 37 ° C for both strains (RF94 and H37Rv) of M. *tuberculosis* and 30°C for the strain (02003) of *M. ulcerans.*

Influence of aqueous extract of P. *amarus* on the *in vitro* growth of two strains of *M. tuberculosis*

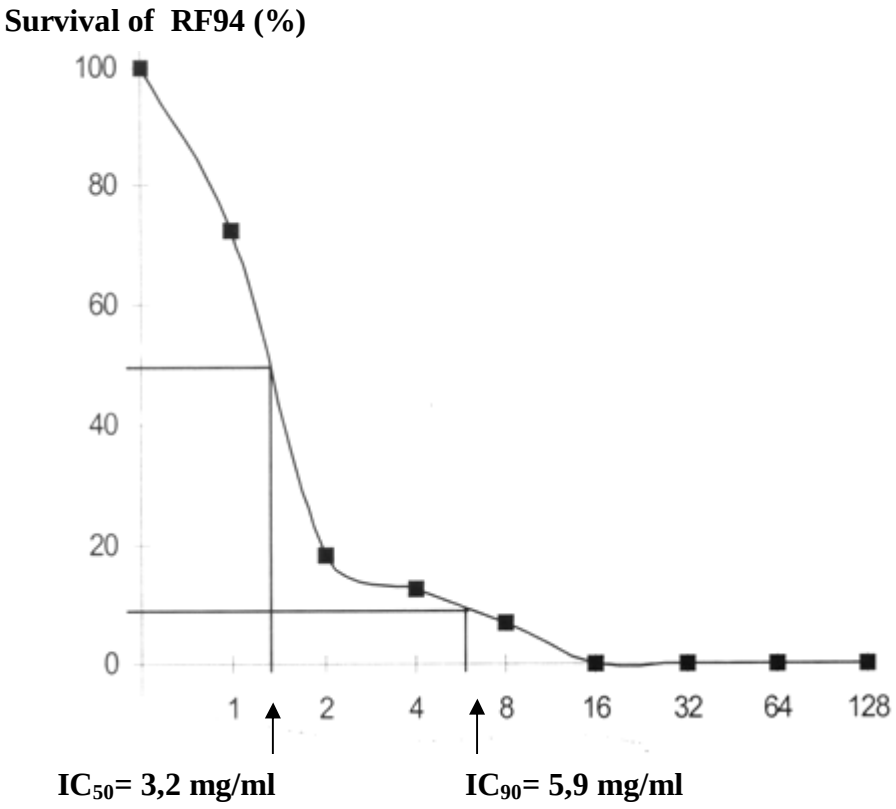

Figure 1 : **Influence of aqueous extract of P. *amarus* on the *in vitro* growth of the RF94 strain of M. *tuberculosis* at 10^{-3} dilution**

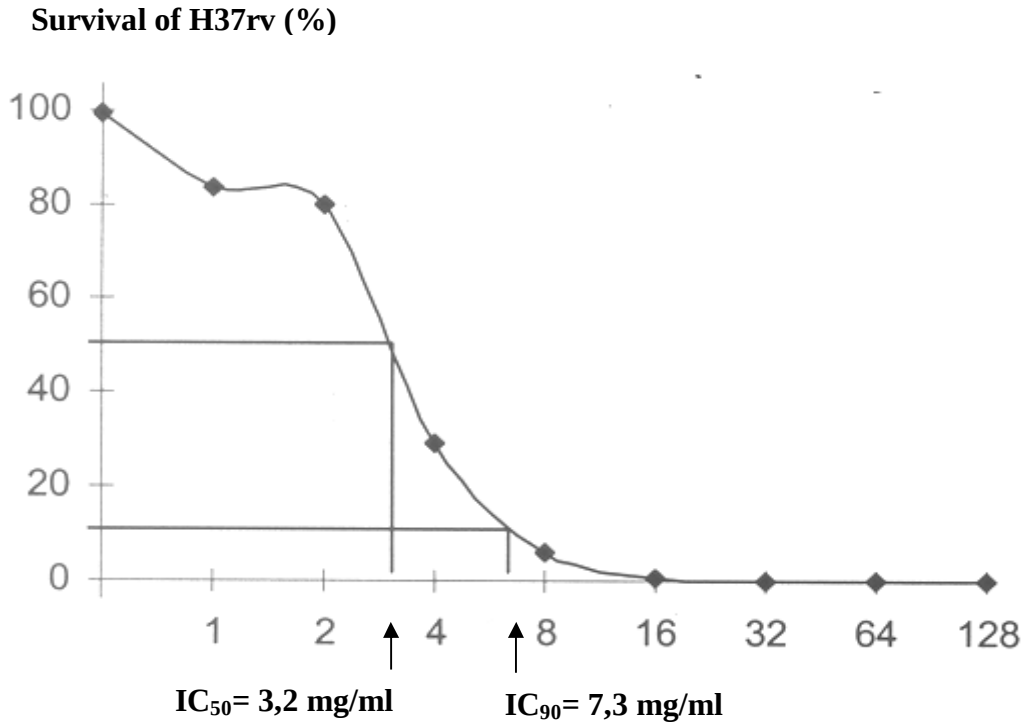

Figure 2 : Influence of aqueous extract of P. *amarus* on the *in vitro* growth of the H37rv strain of M. *tuberculosis* at 10^{-3} dilution

"Fig. 1" and "Fig. 2" represent the antimycobiogrammes obtained from the colony counts value of the control tubes and experimental tubes, concentrations ranging from 0 to 128 mg / ml. These curves represent the survival of the bacteria as the concentration of plant extract increases. Curves disappear at 16 mg / ml and 64 mg / ml. These values are the minimum inhibitory concentrations (MIC). These are the concentrations of the plant aqueous extract where bacteria survival is counted 1% compared to the control tubes respectively for RF94 and H37Rv strains. These MIC values are also equal to the MBC for the two strains of *M. tuberculosis* (Table). The IC_{50} and IC_{90} values were determined graphically and are 3.2 mg / ml and 7.3 mg / ml for H37Rv strains and 1.4 mg / ml and 5.9 mg / ml for the RF94 strain.

Influence of aqueous extract of P. *amarus* on the *in vitro* growth of the 02003 strain of *M. ulcerans*

"Fig. 3" shows the antimycobiogramme of plant aqueous extract concentrations (0-128 mg / ml) based on the survival of bacteria. As the plant extract concentration increases, the value of bacteria counted in the test tube decreases until no longer observed at concentration of 64

mg / ml. This value represents the MIC which is also equal to the MBC. The IC_{50} and IC_{90} values were determined graphically and are 3.5 mg / ml and 19.8 mg / ml for 02003 strains of *M. ulcerans* (Table).

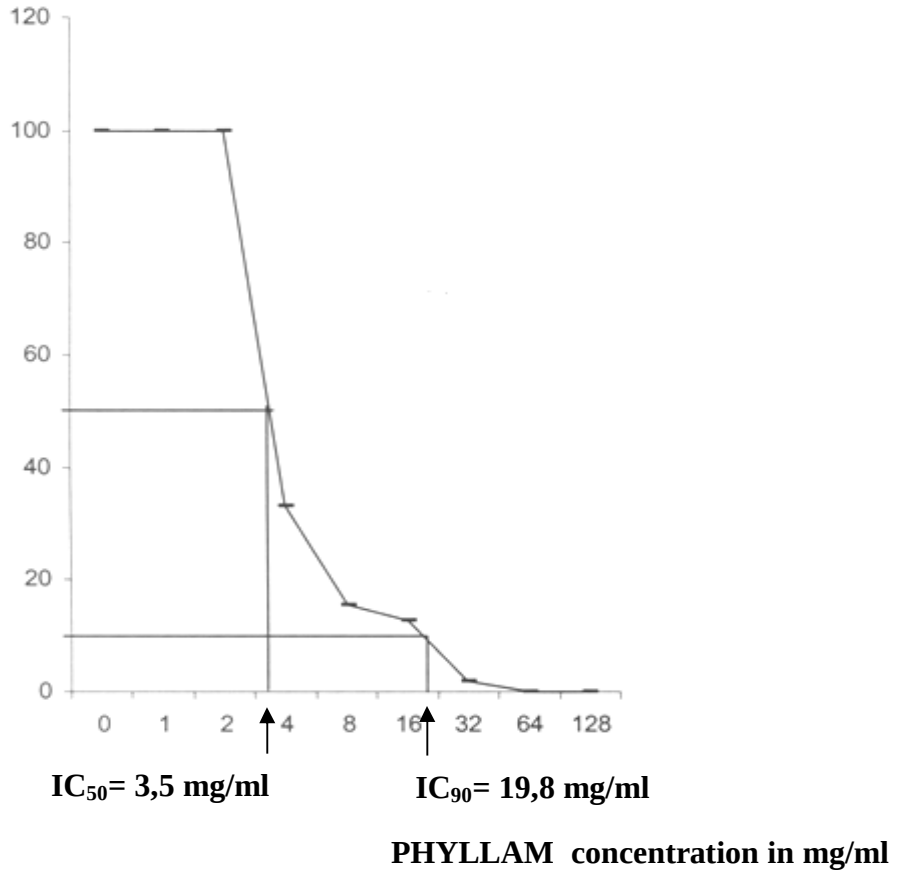

Figure 3: **Influence of aqueous extract of P. *amarus* on the *in vitro* growth of the 02003 strain of M. *ulcerans* at 10^{-3} dilution**

Table 1: Values in mg/ml of antibacterial parameters obtained with the three mycobactaerial strains at 10^{-3} dilution

Strains at 10^{-3} dilution	**Antibacterial parameter**			
	IC_{50}	**IC_{90}**	**MIC**	**MBC**
H37Rv phenotype Inh^S Rif^S	3,2	7,3	64	64
RF94 phenotype Inh^R Rif^R	1,4	5,9	16	16
02003	3,5	19,8	64	64

DISCUSSION

The aim of our study is to determine the antimycobacterial activity of the aqueous extract of a plant coded PHYLLAM (*Phyllanthus amarus*), used in African medicine to treat many diseases [6, 18]. The effect of aqueous extract of PHYLAM (*Phyllanthus amarus*) was tested on the *in vitro* growth of two strains of *M. tuberculosis* (H37Rv and RF94) and one strain of *M. ulcerans* (02003) . The results showed that the three strains of mycobacteria were sensitive to the aqueous extract of *P. amarus*, in a dose-effect relationship; this has resulted in a decreasing pace in all sensitivity curves corresponding to the gradual decrease in the number of survivors of bacteria as the concentration of the aqueous extract of *Phyllanthus amarus* increases. After 8 weeks of incubation, there was a bactericidal action of the aqueous extract at concentrations of 16 and 64 mg / ml respectively for (RF94 and H37rv) strains of *M. tuberculosis* and 64 mg / ml for (02003) strains of *M. ulcerans.* For *M. tuberculosis* strains, we obtained a total inhibition of the growth of viable bacilli from the following concentrations:

MBC (RF94) = 16 mg / ml

MBC (H37rv) = 64 mg / ml

These results showed that the aqueous extract of *Phyllanthus amarus* is more active on RF94 than H37Rv. The effectiveness of *Phyllanthus amarus* on these two strains on the basis of minimum bactericidal concentrations gave:

MBC (H37Rv) / MBC (RF94) = 64/16 = 4

This shows that the aqueous extract is four times more active on RF94 than H37rv. These results are in agreement with those obtained by Adebo in 2000 on the antimycobacterial activity of some plant substances like *Phyllanthus amarus* in Côte d'Ivoire [18, 19]. The results showed that the aqueous extract of *Phyllanthus amarus* is more active on (RF94 and H37rv) strains of *M. tuberculosis* than on (02003) strains of *M. ulcerans*. The reports on the basis of concentrations of 90% inhibition (IC_{90}) are:

IC_{90} (02003) / IC_{90} (RF94) = 19.8 / 5.9 = 3.4

IC_{90} (02003) / IC_{90} (H37rv) = 19.8 / 3 = 2.7

The aqueous extracts of *Phyllanthus amarus* is 3.4 times more active on RF94 and 2.7 more active on the H37rv strain than 02003 strain of *M. ulcerans.*

Comparison of inhibitory concentrations for 50 % survival (IC_{50}) gave: IC_{50} (02003) > IC_{50} (H37rv) > IC_{50} (RF94).

These results confirm that the strain (RF94) of *M. tuberculosis* is more sensitive to the aqueous extract of *Phyllanthus amarus* than the other two strains. Indeed, it should be noted that the activity of a plant material depends on several factors, including the part of the plant used (leaves, stems and roots), the method of extraction and concentration of the active molecule [20]. In our study, we used all parts of the plant (leaves, stems and roots) and our study showed that our extraction method is better in terms of concentrating active molecule.

Several studies carried out on the anti -infective activity of medicinal plants have shown that the activity of aqueous extracts can be improved by fractionation using chromatographic techniques that can target the active fractions [16]. Our results suggest that purification should be done or fractionation to enhance performance.

CONCLUSION

We tested different concentrations of the aqueous extract of PHYLLAM on 02003 strain of M *ulcerans* and H37rv, RF94 strains of *M. tuberculosis*. PHYLLAM has bactericidal activity in vitro against the three strains studied. A dilution of 10^{-3} , PHYLLAM completely inhibits the growth of bacteria from the concentration of 16 mg / ml for RF94 , 64 mg / ml for H37Rv and 02 003. Comparing concentrations for 90 % inhibition CI_{90}, it appears that PHYLLAM is 3.4 times more active on RF94 and 2.7 times more active on H37rv strain than 02003 strain of *M ulcerans.*

Our work could be an important step in the fight against Buruli ulcer and relieve many patients who hope for a cure other than surgery. It should therefore be continued with a wider range of sample to be tested, *in vitro* and *in vivo.*

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