International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2017): 7.296

In vitro Evaluation of the Antibacterial Activity of Extracts of *Phyllanthus amarus* on Three Germs (*Escherichia coli*, *Pseudomonas aeruginosa and Staphylococcus aureus*) of Superinfection of Buruli Ulcer in Côte d'Ivoire

Bakary Coulibaly[1], Ibourahema Coulibaly[2], Kouassi Kouassi Clement[3], Sylvia Monthaut[4], Joseph Allico Djaman[5]

[1, 2, 3, 4, 5]Training Unit and Research in Agroforestry, Laboratory of Microbiology and Food Engineering (LMFI), University Jean Lorougnon Guédé, BP. 150 Daloa, Ivory Coast.

Abstract: ***Complications of Buruli's ulcer associated with bacterial superinfections make it difficult to manage this infection. In addition, the rising costs of treating infections caused by resistant bacteria call for alternative care alternatives. The development of new antibacterial molecules is essential to fight against these scourges. It is for this purpose that this study was carried out by evaluating the activity of aqueous and ethanolic extracts of P. amarus on the in vitro growth of three Buruli ulcer superinfection organisms in Ivory Coast. The methods of diffusion in agar medium and in liquid medium were used for the sensitivity test and the determination of the MIC and the CMB. The different tests were performed on three isolated strains of patients with Buruli ulcer. The minimum inhibitory concentrations of the extracts vary between 5 mg / ml and 20 mg / ml and the minimum bactericidal concentrations between 10 mg / ml and 40 mg / ml. The lowest value of MIC and CMB was observed with S. aureus637 while the highest value of these same parameters was obtained on E. coli 250. These extracts exerted a bactericidal activity on P. aeruginosa, S. aureus, E. coli. This could justify the use of this plant in the treatment of various infections in traditional environments.***

Keywords: M. ulcerans, E. coli, P. aeruginosa, S. aureus, P. amarus, antibacterial activity, aqueous extract, ethanolic extract, MIC, CMB, Buruli ulcer,

1. Introduction

Buruli ulcer (BU) is a disease caused by a slow-growing bacterium called Mycobacterium ulcerans. The disease manifests itself in various forms. The most spectacular being large skin ulcers that lead to disabling sequelae [1]. Most of the complications are related to bacterial superinfection and all the germs can be found in the ulcer [2]. The bacteria most involved in the occurrence of these superinfections are: *P. aeruginosa* with 28.57% of cases of infection, Proteus mirabilis (28.57%), *Klebsiella pneumoniae* (17.86%), *Escherichia coli* (14.29%) %) and *S. aureus* (with 22.4 to 50% of superinfection cases) according to studies conducted by [3]. The same authors demonstrated that 96.80% of bone disorders are related to bacterial superinfection. They have also shown that the weight loss of patients, a fever or a very advanced anemia is linked to a major superinfection. Thus bacterial superinfections can progress to phlegmon or sepsis [4]. [5] has shown in a similar study that 16% of underlying osteomyelitis caused by resistant *M. ulcerans* present superinfection. These complications make it difficult to manage the infection.

In view of this, the search for new molecules is needed to ensure the management of bacterial superinfection of BU against therapeutic failures and the rising costs of treating infections caused by resistant bacteria. Traditional medicine, which has often been a source of evidence for active ingredients, could be a potential source of new molecules against these bacteria.

Thus *P. amarus*, an herbaceous plant, was chosen for this study for its multiple therapeutic properties according to the scientific investigations carried out in Latin America and in the Indian subcontinent. Indeed, different properties of this plant have been highlighted. These include antiviral properties [6] in the treatment of viral hepatitis, antifungal, antibacterial properties [7] and antimycobacterial properties [8].

The general objective of this study is to highlight an antibacterial activity of *P. amarus* against the superinfections of Buruli ulcer in a context of valorization of traditional Ivorian medicine.

- The specific objectives:
- Prepare the aqueous and ethanolic extracts 70% of *p. Amarus*
- Determine the inhibition diameters of these extracts in a solid medium.
- Determine the antibacterial parameters (mic and cmb).

2. Material and Methods

2.1 Materials

2.1.1 Preparation of the plant extract

The plant material consists of powder obtained from dried whole plants of *P. amarus*. These plants were collected in

Daloa in the Haut-Sassandra region of west-central Côte d'Ivoire in September 2016.

2.1.2 Bacterial material

The bacterial material consists of *E. coli* 250, *E. coli* strain, *P. aeruginosa* SB17 strain of *P. aeruginosa* and *S. aureus* 637 strain of *S. aureus*, all isolated from pus in patients with BU in the Bacteriology-Virology laboratory of the Regional Hospital Center (CHR) in Daloa

2.2 Methods

2.2.1 Preparation of the plant extract

The plants were washed, sorted and then dried in the laboratory at room temperature for six weeks. After drying, they were ground using a Retsch SK100 grinder. A brown powder is obtained. This powder was used for the preparation of 70% aqueous and ethanolic extracts according to the method described by [9].

2.2.2 Determination of the antibacterial activity of plant extracts

Preparation of the concentration range

The preparation of the concentration range was made according to the method of [10]. A stock solution of concentration 400 mg / ml was prepared in sterile distilled water with the dried extracts of *P. amarus*. This solution was subjected to a dilution series of reason 2 which made it possible to obtain concentrations ranging from 200 to 12.5 mg / ml.

Preparation of bacterial inocula

The method of [10] was used for the preparation of bacterial inocula. A young colony 18-24 hours of each bacterial culture is removed using a loop of platinum and then homogenized in 2 ml of 85% NaCl suspension. Then, the optical density was adjusted to 0.5 Mac Farland. A volume of 100 µl of this suspension was diluted in 10 ml of physiological saline (0.9% NaCl). This bacterial suspension is evaluated at approximately 106 cells / ml and constitutes the pure inoculum.

Sensitivity test (determination of inhibition zone diameters)

The sensitivity of the strains to plant extracts was achieved by the diffusion technique in agar medium. Mueller Hinton habitats were inoculated by flooding. Using a sterile punch, wells about 6mm in diameter were made in the agar plate. Each well received 80µl of the test substance at concentrations 200, 100, 50, 25 and 12.5 mg / ml. After 30 minutes of diffusion at laboratory temperature, the petri dishes were incubated at 37 ° C. for 18 to 24 hours. The presence or absence of an inhibition zone has been observed.

The interpretation of the results was performed according to the criteria defined by [11]. According to these authors, a substance has no activity if the diameter of zones of inhibition (ZI) <5 mm; has low activity if $5 \leq ZI < 8$ mm; has an average activity if $8 \leq ZI < 12$ mm and a high activity if $ZI \geq 12$ mm.

2.2.3 Determination of antibacterial parameters

Preparation of inocula

Bacterial inocula were prepared according to the method of [10] from colonies younger than 24 h in Mueller Hinton Broth (BMH). An isolated colony of each bacterial culture was removed using a platinum loop and diluted in 10 ml of BMH and incubated for 3 to 5 hours at 37 ° C. A volume of 0.1 ml of the different pre-cultures was taken respectively and then added to 10 ml of sterile BMH. This bacterial suspension constituted 100 dilution or pure inoculum and is evaluated at about 106 cells / ml.

Inoculum count

The method of [12] was used to count the inoculum. A 10 to 10 dilution of BMH inoculated to 10-4 was performed. These different dilutions as well as the pure inoculum were inoculated with a loop calibrated by streaks 5 cm long on a Mueller Hinton agar (GMH) and incubated at 37 ° C for 24 h. This series of preparation constituted box A.

2.2.4 Preparation of the concentration range of plant extracts

The concentration range was prepared with sterilized extracts autoclaved at 121° C for 15 minutes. It was prepared in eight (8) test tubes by the double dilution method in a geometric progression of reason 2 with concentrations ranging from 160 mg / ml to 1.25 mg / ml [12].

Inoculation

For carrying out this test a series of 10 hemolysis tubes numbered from C_1 to C_{10} was used. The tube numbered C_{10} constituted the sterility control of the extract which contained 1 ml of distilled water and then 1 ml of extract. The tube numbered C_9 is the indicator of growth of germs. In this tube was introduced 1ml of sterile distilled water and 1ml of inoculated broth. The tubes C_1 to C_8 received 1ml of concentrated BMH twice inoculated and 1ml of plant extract of well-known concentration according to the concentration range prepared. This distribution of plant extract was made so that 1ml of vegetable extract of 160 mg / ml is transferred to tube C_1 and tube C_8 received 1 ml of plant extract of 1.25 mg / ml. Due to the volume / volume dilution achieved, the concentration in the tubes was reduced by half. Thus the concentration of the C_1 tube was increased from 160 mg / ml to 80 mg / ml so on until the C_8 tube which passes from 1.25 mg / ml to 0.62 mg / ml. After inoculation, all tubes were incubated at 37°C for 18-24 h. The experiment was done three (3) times.

Determination of the minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of the substance for which there is no visible growth with the naked eye after an incubation time of 18 to 24 hours. His determination was made by observation of the disorder induced by the growth of the germs studied in each tube.

Determination of the minimum bactericidal concentration (CMB)

The minimum bactericidal concentration (MBC) is the lowest concentration of substance that leaves at most 0.01% of surviving germs. Using a loop calibrated at 2 µL, the

contents of the tubes in which no haze was observed were removed and seeded on a Mueller-Hinton agar, starting with the tube of the MIC. Seeding was done by parallel streaks 5 cm long at the agar surface (Box B). After 24 hours' incubation in an oven at 37 ° C, the number of colonies on the streaks was compared with those of the inoculum counting box (Box A). Thus, the first experimental tube whose number of seeds present on its streak is less than or equal to that of the 10-4 dilution will correspond to the CMB. The antibacterial effect has been determined to be bactericidal or bacteriostatic depending on the ratio: CMB/MIC. Indeed, if CMB/MIC ≤ 4, the effect is bactericidal and if CMB / MIC> 4, the effect is bacteriostatic [13].

3. Results

3.1 Sensitivity tests (inhibition zone diameters)

Susceptibility testing of P. amarus aqueous extract (XA)

The results recorded in Table II showed that the aqueous extract had a good inhibitory activity at the different concentrations tested on the bacterial strains with a diameter of inhibition of 14.33 mm on *S. aureus* 637 at 25 mg / ml per concentration. However, a very low sensitivity was observed with *E. coli* strains. *E. coli* 250 and *P. aeroginosa* SB17 with respective inhibition diameters of 0 mm at this same concentration (**Table 2**). This good activity is observed rather at the concentration of 50 mg / ml for *P. aeroginosa* SB17 with an inhibition diameter of 14.66 mm and at a concentration of 100 mg / ml with an inhibition diameter of 18.33 mm for *E. coli* 250.

Sensitivity tests with P. amarus ethanol extract 70% (XE)

The results shown in Table III show that the 70% ethanolic extract has a good inhibitory activity on bacterial strains at varying concentrations from one strain to another. Thus, this activity is observed on the strain of *S. aureus* and *P. aeroginosa* at the concentration of 50 mg/ml with respective inhibition diameters of 18.33 mm and 14.66 mm. As for *E. coli*, the activity is observed at a concentration of 100 mg / ml with an inhibition diameter of 18.33 mm (**Table 3**).

Sensitivity tests with P. amarus ethanol extract 70% (XE)

The results shown in Table III show that the 70% ethanolic extract has a good inhibitory activity on bacterial strains at varying concentrations from one strain to another. Thus, this activity is observed on the strain of *S. aureus* and *P. aeroginosa* at the concentration of 50 mg / ml with respective inhibition diameters of 18.33 mm and 14.66 mm. As for *E. coli*, the activity is observed at a concentration of 100 mg / ml with an inhibition diameter of 18.33 mm (**Table 3**).

4. Discussion

This study involved the extraction with water and ethanol of 70% of the whole plant of *P. amarus* and to evaluate the antibacterial activity of these extracts on the in-vitro growth of bacterial strains involved in superinfection of Buruli ulcer. It is situated in a context of valorization of the Ivorian pharmacopoeia as most patients prefer self-medication and the use of traditional treatment. Inpatient treatment is only a last resort. All bacterial strains namely *S. aureus* 637, *P. aeroginosa* SB67 and *E. coli* 250 tested are sensitive to both extracts of *P. amarus* which resulted in a decrease in the disorder caused by the growth of germs in the experimental tubes. as the concentration of extract increases compared to the growth control.

Our results show that 70% aqueous and ethanolic extracts had antibacterial activity by inhibiting sprout growth in a dose-response relationship. This allowed us to determine the different antibacterial parameters namely the MIC and the CMB. Strains of *S. aureus* 637 were more sensitive to the aqueous extract with MIC and CMB values of 5 and 10 mg/mL. As for the sensitivity to 70% ethanolic extract, *P. aeruginosa* strain SB67 is the most sensitive with a MIC and a CMB of 5 and 10 mg/ml. The highest values of MIC and CMB were observed with the strain of *E. coli* 250 with both extracts. A similar study was conducted by [14] and [15].

In fact, they noted an antibacterial activity of the aqueous and ethanolic extracts 70% of the leaves of plants vis-a-vis strains of *S. aureus*, *P. aeruginosa*, *E. coli* and *S. Typhi*. These results are in agreement with those of [16] who found that *S. aureus* and *P. aeruginosa* were more sensitive than E. coli to the aqueous extract of the trunk of *Harungana madagascariensis* at concentrations ranging from 50 mg / ml to 100 mg / ml with inhibition zone diameters ranging from 11 mm to 18 mm, compared to 0 mm for *E. coli*. On the other hand, these results are in contradiction with those of [17]. Indeed, these authors have demonstrated that the aqueous extract of *P. amarus* has a better activity on *E. coli* and *P. aeruginosa* by inducing diameters of 18 mm inhibition zones on these two strains than on *S. aureus* with an inhibition zone diameter of 17 mm at the concentration of 50 mg/ml. In order to improve the antibacterial activity of the aqueous total extract (EA), we used a 70/30 ethanol / water solvent mixture to prepare another extract. We called it ethanol extracts 70%. The comparison of the antibacterial parameters of the aqueous extract and the 70% ethanolic extract indicates a difference for all the bacterial strains studied.

The MIC ratio shows that the 70% ethanolic extract of *P. amarus* is twice as active on *P. aeruginosa* SB67 and *E. coli* 250 strains compared to the aqueous extract. However, this report indicates that the aqueous extract is twice active on *S. aureus* 637 than the ethanol extract 70%. The CMB / MIC ratios for all strains and for both extracts are equal to 2.

It follows from our analysis that the aqueous and ethanolic extract 70% of *P. amarus* have a bactericidal power on the strains studied.

5. Conclusion

From this study, which aimed to evaluate in vitro the antibacterial activity of the aqueous and ethanolic extracts of 70% of the whole plant of *P. amarus* on superinfection germs of Buruli ulcer, it appears that:

- All the strains studied were sensitive to the aqueous and ethanolic extract 70% of *P. amarus*.

- The determination of the sensitivity of the strains to the different extracts depends on the concentrations of the extracts;

Both extracts exerted a bactericidal power on the different strains.

The sensitivity of the different strains to *P. amarus* extracts is of great importance in the treatment of Buruli ulcer secondary infections, some of which have high resistance to antibiotics used in routine practice.

This study justifies the merits of the antimicrobial virtues granted to this plant. This will lead to an improved therapeutic arsenal based on plant for the benefit of patients with Buruli ulcer.

References

[1] Guédénon A., Portaels F., Aguiar J., Cissé L., Debacker M., Meyers W.M., Zinsou C. **"**Ulcère de Buruli (Infection à *Mycobacterium ulcerans*). Guide diagnostique à l'usage des professionnels de la santé". p5, 2000.

[2] Richard-Kadio M. , Dick R. , Djeha D. , Eloiflin B. , Kossoko K. , Djemadje N. , Casanelli J. M. , N'guessan H. A. , Heroin P. , Cornet L. "Les ulcères cutanés a mycobactéries atypiques et leur traitement par la chirurgie plastique". *Médecine d'Afrique Noire*., **37** (5), **pp** 276- 282, 1990.

[3] Ecra E. , Yoboue P. , Aka B. , Gbery I. , Sangare A. , Kanga K. , Cisse M. , Bamba V., Djeha D. et Kanga JM. **"**Les complications de l'ulcère de buruli : analyse de 97 CAS". *Médecine d'Afrique Noire* - **48** (4)**,** pp154-158, 2001.

[4] Portales F., Paul Johnson et Waynem Meyers., "Ulcère de Buruli: Diagnostic de l'infection à *Mycobacterium ulcerans* WHO/CDS/CPE/GBUI/2001".**4** p 4, 2001.

[5] Lagarrigue V., **"**L'ulcère de Buruli avec atteinte osseuse : à propos de 33 cas au Bénin". *Med. Trop*. **59** (3); p 312, 1999.

[6] Thyagarajan S.P., Subramanian S., Thirunalasun D T. **"**Effects of *Phyllanthus amarus* on chronic carriers of hepatitis B virus". *Lancet*, **6** p **764**, 1988

[7] M'baye A L. **"**Evaluation de l'activité antifongique *Cassia alata*, de Misca et de *Phyllanthus amarus* sur la croissance *in vitro* de *Aspergillus flavus"*. DEA, Université de Cocody, Abidjan 35p.

[8] Coulibaly B., N'guessan K. R., AKA N., Ekaza E., N'golo D. C., Trébissou N., Ouattara L., Bahi C., Coulibaly A., Assandé J. M., Mohui P., Yao H., Djaman A. J., & Dosso M., Activité anti-mycobactérienne *in vitro* des extraits de *Phyllanthus amarus* (Schum et Thonn) sur les souches de *Mycobacterium ulcerans* en Côte d'Ivoire. *Bulletin de la Société Royale des Sciences de Liège*, **80**, pp 759 – 771. 2011.

[9] Zirihi G. N., Kra A. K. M. ., Guede-Guina F. **"**Evaluation de l'activité antifongique de *Microglossa Pyrifolia* (Asteraceae) sur la croissance *in vitro* de *Candida albicans"*. *Revue de médicines et Pharmacopées Africaines*, **17**: pp 11-18. 2003.

[10] Bssaibis F., Gmira N. et Meziane M**.,** **"**Activité antibactérienne de *Dittrichia viscosa* (L.)" W. Greuter. *Rev. Microbiol. Ind. San Environn*., **3**: pp 44-55, 2009

[11] Konan K. F., Guessennd K. N., Oussou K. R., Bahi C., Coulibaly A., Djaman A. J. et Dosso M. **"**Effet antibactérien de l'extrait aqueux de l'écorce de *Terminalia glaucescens* Planch ex Benth (Combretaceae) sur la croissance *in vitro* des entérobactéries productrices de bêta-lactamases à spectre élargi (EBLSE)" *Int. J. Biol. Chem. Sci.* **8**(3) pp 1192-1201, 2014.

[12] Kanga Y., Guessennd N., Gnahoue G., Bene K., Zirihi-Guédé N., et Dosso M. "Etude botanique et évaluation de l'activité antibactérienne de l'extrait aqueux de *Hunteria eburnea* Pichon (Apocynaceae) sur la croissance *in vitro* de souches multi-résistantes isolées chez des patients hospitalisés dans un CHU en Côte d'Ivoire". *International Journal of Innovation and Scientific Research*. **21** (1), pp. 154-161, 2016.

[13] Marmonier A. A**.** "Introduction aux techniques d'étude des antibiotiques". *In* Bactériologie Médicale, techniques usuelles, pp 227-236, 1990

[14] Madubunyi I. I., Obi S. K. C., Nwebube N. I., and Chime A. B., "Antihepatotoxic and antimicrobial activities of Harungana madagascariensis leaf exracts". *Int. J. Pharmacol.*, **33** (2), pp 129-134, 1995,

[15] Okoli A. S., Okeke M. I., Iroegbu C. U., and Ebo P.U**.,** "Antimicrobial activity of *Harungana madagascariensis* leafs extracts". *Phythérapy Research*, 16, pp 174-179, 2002.

[16] Babatunde S. K., Abubakare A. A., Abdulraheem Y .J. et Ajiboye E .A. Antimicrobial activity of *Phyllanthus amarus* on some human intestinal facultatively anaerobic flora International Journal of Medicine and Biomedical Research**,** 23 (2) pp 120-124, 2014

[17] Toty A. A., Guessennd N. , Bahi C. , Kra A. M. ,Otokore D. A. et Dosso M. "Evaluation *in vitro* de l'activité antibactérienne de l'extrait aqueux de l'écorce de tronc de *Harungana madagascariensis* sur la croissance de souches multi- résistantes". *Bulletin de la Société Royale des Sciences de Liège, V 82, pp12-21***,** 2013