Buruli ulcer (BU), caused by the environmental organism *Mycobacterium ulcerans* and characterized by necrotizing skin and bone lesions, poses important public health issues as the third most common mycobacterial infection in humans [1]. The disease has become substantially more frequent over the past decade, particularly around the Gulf of Guinea, and has been detected or suspected in at least 31 countries. Clinical diagnosis of BU disease should be confirmed by PCR, as recommended by the World Health Organization (WHO), and case patients should be treated with rifampin/streptomycin
daily for 8 weeks (therapy available since 2004), combined, if necessary, with surgery.

In Benin, sociocultural beliefs and practices strongly influence the health-seeking behaviours of people affected by BU. The first recourse is often traditional treatment. Most of the components in the traditional treatment belong to the plant kingdom [2]. Plants provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Recently we carried out an ethnobotanical survey involving seventeen traditional practitioners within the Ouinhi community in the Zou Department (Benin). We noted that about forty-nine different plants were used for the traditional treatment of BU. Different parts of these plants were included in various pharmaceutical forms for internal or external use [2]. We realized a screening of 44 plant extracts used in traditional medicine to treat BU [2, 3]. Results showed that crude hydroethanolic extract of Holarrhena floribunda was effective in inhibiting the growth of Mycobacterium ulcerans (MIC = 0.075 g/L) and other microorganisms [10, 11].

Holarrhena floribunda (G. Don) T. Durand and Schinz grows as a shrub or tree up to 25 m tall, with a stem diameter of up to 30 cm. Its fragrant flowers feature a white corolla. Fruit is pale grey to dark brown with paired follicles, each up to 60 cm long. This species is known as four synonyms: Rondeletia floribunda G. Don, Holarrhena africana A. DC., Holarrhena wulfsbergii Stapf, Holarrhena ovata. In the Republic of Benin, the common names of this plant are: Fon: “kpakpatoun” [2]; Yoruba and Nago: “ako ire,” “ire Ibedji;” Mina: “gaoti” [4].

Here we describe compounds identified from bioactive fractions of the extract and evaluation of the inhibitory effect of one of them on the growth of M. ulcerans.

2. Materials and Methods

2.1. Experimental

2.1.1. Plant Materials. Plant species was collected and identified by a botanist from the National Herbarium of Benin and voucher specimens (Yemoa 06) are deposited at the same herbarium.

2.1.2. Preparation of Crude Hydroethanolic Extracts. Dried plants were ground to a powder with a pulverizator (National Mixer Grinder Mx-119N, Japan). 50 g of powder was then macerated 48 h (at room temperature) in 70% ethanol in a 1/10 (w/v) ratio. The material was filtered through a Millipore filter of 0.2 µm (Acrodisc, USA). The filtrate was concentrated under reduced pressure at less than 40 °C using a rotary evaporator (Buchi Rotavapor R-200/205, Switzerland) to obtain a crude residue.

2.1.3. Fractionation of Crude Extracts and Isolation of Active Constituents. Crude hydroethanolic extract was fractionated on silica gel 60 (0.063–0.200 mm Merck, Germany) by atmospheric pressure liquid chromatography eluting with solvents of increasing polarity, namely, hexane, dichloromethane, ethyl acetate, and water, yielding 4 fractions (F1, F2, F3, and F4). Of these, fraction F2 (dichloromethane) was found to cause growth inhibition of M. ulcerans and was, as a result, selected for further work. This fraction was monitored using TLC plates (TLC silica gel 60 F254, Merck) and led to characterize the presence of alkaloids (toluene : ethyl acetate : diethylamine: [7:2:1], detection-Dragendorff’s spray reagent). We then performed a more specific extraction to obtain an enriched alkaloid extract (Figure 2).

We therefore proceeded to fractionation of this enriched extract by Atmospheric Pressure Liquid Chromatography (APLC) and Medium Pressure Liquid Chromatography (MPLC) followed by gel filtration on Sephadex LH20 to purify this enriched alkaloid fraction.

Atmospheric Pressure Liquid Chromatography (APLC). Alkaloids enriched extract was repeatedly fractionated on silica gel 60 (0.063–0.200 mm, Merck, Germany) eluting with solvents of increasing polarity (mixture of CH2Cl2/MeOH 50:50 to 100% MeOH followed by MeOH : H2O 90:10 and MeOH : acetic acid 90:10). Fractions were monitored by TLC (toluene : ethyl acetate : diethylamine: [7:2:1], detection-Dragendorff’s spray reagent) and similar fractions were combined and concentrated in vacuo. Prepurified fractions obtained by APLC were rechromatographed by MPLC.

Medium Pressure Liquid Chromatography (MPLC). MPLC was performed on glass columns packed with LiChroprep Si 60 (15–25 µm) from Merck with a mobile phase composed of CH2Cl2/MeOH 50:50 to 100% MeOH. Prepurified fractions obtained were monitored by TLC (toluene : ethyl acetate : diethylamine: [7:2:1], detection-Dragendorff’s spray reagent) and similar fractions were combined and concentrated in vacuo followed by gel filtration on Sephadex LH20 (MeOH). Five different purified fractions were obtained and analyzed by high pressure liquid chromatography coupled to mass spectroscopy (HPLC-MS or LC-MS) (Thermo Scientific Accela LC Systems, orbitrap).

LC-MS. High pressure liquid chromatography coupled to a diode array detection and mass spectrometry with positive electrospray ionization (HPLC-ESI-MS³) was employed to rapidly separate and identify the constituents in these five purified fractions. The LC MS/MS system consisted in a Thermo Accela pump, autosampler, photodiode array detector, and Thermo Scientific LTQ orbitrap XL mass spectrometer.
3. Results and Discussion

Our previous investigations allowed determining that the hydroethanolic extract of *H. floribunda* inhibited growth
of *M. ulcerans* with a MIC value of 125 mg/mL using the resazurin microtiter assay (REMA) [3]. The CH$_2$Cl$_2$ fraction of this extract also inhibited growth of *M. ulcerans* with a MIC value of 125 mg/mL and contained alkaloids. Fractionation of an enriched alkaloid extract (MIC = 62.5 mg/mL) was undertaken as described in the Experimental Section, allowing the identification of four different alkaloids in five different fractions and isolation of one of them with high purity. The structure of the major compound was identified as holadysamine (3-methyl amino pregn5,16-dien-20-ol) by MS and NMR [8]. LC-MS was used to identify alkaloids in the other fractions, named compounds A, B, and C, and gave molecular formula of, respectively, C$_{22}$H$_{37}$NO (MW: 331.29), C$_{23}$H$_{33}$ON (MW: 315.26), and C$_{23}$H$_{37}$ON (MW: 343.29). According to bibliographic data on compounds isolated from *H. floribunda* [7, 8], they could correspond to holaphyllinol (MW: 331.29), holamine, or holaphyllamine (MW: 315.26), N,N-dimethyl holamine, N-dimethyl holaphyllamine or methyl 6 holaphylline (MW: 343.29). HPLC-ESI-MS/MS allowed us to show that compound A could correspond to holaphyllinol and B to holamine or holaphyllamine while C seems to be a new compound. On the basis of the analysis of spectroscopic data, we observed the presence of a fragment at m/z 326 ([M-H$_2$O+H]$^+$) and the loss of a water molecule in the MS/MS fragmentations of C allowing us to propose an alcoholic and not a ketonic function on C20 (Figure 3).

Further studies must be performed to confirm these chemical structures. It could not be done because of the lack of compound.

Fraction containing pure holadysamine was found to be more active (MIC = 50 mg/mL) than fractions containing A, B, or C or the crude hydroethanolic extract (125 mg/mL) but less active than the reference rifampicin (MIC = 2 mg/mL). This low activity could not totally explain the use of this plant in traditional treatment but as this plant is used in mixtures with other plants; the various compounds present in these plant extracts can act synergistically as it is the case with the antibiotics ethambutol, clarithromycin, and rifampicin [12]. Rastogi and Labrousse in their study showed that the use of these antibiotics in combination results in increased bactericidal effects compared to drugs used alone or in combination with two of them.

**4. Conclusions**

Our results show that extracts of aerial parts of *H. floribunda*, used by traditional healers to treat BU, exhibited significant in vitro antimycobacterial activities. Four active alkaloids have been identified. Holadysamine the major compound was found to be the most active one (MIC = 50 mg/mL), but this activity is lower than that of rifampicin. As synergy may be found between different compounds, it would be interesting to analyze the efficacy of the other alkaloids and their combinations. Furthermore, as this plant is used in association with other ones, it is also interesting to test plant associations as used by traditional practitioners. Remedies that would prove effective activity should be applied for toxicological and pharmacological studies. There is also an urgent need of standardization of traditional remedies based on plants.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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