Antimycobacterial screening of plants from Benin on *Mycobacterium ulcerans*, the causal agent of Buruli ulcer

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Screening antimycobactérien de plantes béninoises sur Mycobacterium ulcerans, agent causal de l'ulcère de Buruli.

Résumé : Nous avons entrepris sur Mycobacterium ulcerans, un screening microbiologique de 44 plantes médicinales sélectionnées parmi 49 plantes utilisées dans le traitement traditionnel de l'Ulcère de Buruli (UB) au Bénin. Différents extraits bruts ont été préparés à partir des feuilles, des fruits, des racines de ces plantes et les Concentrations Minimales Inhibitrices (CMI) des solutions d'extraits de plantes bruts ont été déterminées par la technique REMA (Resazurin Microtiter Assay).

Nous avons montré que les extraits bruts de Holarrhena floribunda (G.Don) T. Durand et Schinz et de Jatropha curcas Linn possèdent in vitro une activité antimycobactérienne intéressante sur M. ulcerans avec des CMI respectives de 125 et 250 µg/ml.

La technique REMA que nous avions adaptée et utilisée dans cette étude pour évaluer l'activité in vitro d'extraits de plantes est une méthode simple, sensible, rapide et pourrait être utilisée comme une méthode de choix pour le screnning antimycobactérien des extraits de plantes sur M. ulcerans. Ceci pourrait contribuer à documenter le traitement traditionnel de l'ulcère Buruli.

Mots clés: *Ulcère de Buruli; Médicine traditionnelle; Plantes médicinales; Activité antimycobactérienne; Bénin.*

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Abstract: The purpose of this study is to investigate the antimycobacterial activity against *Mycobacterium ulcerans* of plants used in traditional medicine in Benin to treat Buruli ulcer (BU). Out of 49 plants identified, 44 were selected and screened. Crude extracts of leaves, fruit seeds, and roots were prepared and the minimum inhibitory concentration (MIC) values were determined using an adapted Resazurin Microtiter Assay (REMA). The crude extracts of *Holarrhena floribunda* (G.Don) T. Durand and Schinz and *Jatropha curcas* Linn showed inhibitory activity against *M. ulcerans* at concentrations of 125 and 250 µg/ml respectively. The adapted REMA method used in this study to determine the MIC values of natural product is simple, sensitive, and rapid, and could be a method of choice to successfully assess antibacterial properties of plants extracts against *M. ulcerans*. This can also contribute to document traditional treatment of BU.

Keywords: *Buruli ulcer; Traditional medicine; Medicinal plants; Antimycobacterial activity; Benin.*

Triagem de plantas com atividade antimicobacteriana originárias de Benin testadas frente ao Mycobacterium ulcerans, o agente causal da úlcera de Buruli.

Resumo: O objetivo deste estudo foi investigar a atividade antimicobacteriana frente ao *Mycobacterium ulcerans* de plantas usadas na medicina tradicional para tratar úlcera de Buruli (UB) em Benin. A partir de 49 plantas identificadas, 44 foram selecionadas e testadas. Extratos brutos de folhas, sementes e raízes foram preparados e a concentração inibitória mínima (CIM) foi determinada pelo ensaio da microdiluição em placa com resazurina (REMA) adaptado para *M. ulcerans*. Os extratos brutos da *Holarrhena floribunda* (G.Don) T. Durand e Schinz e *Jatropha curcas* Linn apresentaram atividade inibitória frente ao *M. ulcerans* nas concentrações de 125 e 250 µg/ml, respectivamente. O método REMA, adaptado para determinar a CIM dos produtos naturais, mostrou-se simples, sensível, rápido e poderia ser um método de escolha para determinar a propriedade antimicobacteriana de extratos de plantas frente ao *M. ulcerans*. Estes achados também podem contribuir para documentar o tratamento tradicional da UB.

Palavras chaves: *úlcera de Buruli; medicina tradicional; medicina plantas; atividade antimicobacteriana; Benin.*

1. Introduction

Buruli ulcer (BU) is a skin disease caused by *Mycobacterium ulcerans*. The disease often occurs in close proximity to aquatic ecosystem (river, natural or artificial lake, swamp, irrigation system). BU has been reported from at least 27 countries around the world, mostly in tropical areas [1]. In Benin, BU is the second mycobacterial disease after tuberculosis, with nearly 7000 cases reported between 1989 and 2006 [2].

It usually begins as a painless nodule or papule and may progress to massive skin ulceration. If untreated, BU may lead to extensive soft tissue destruction that may extend to the deep fascia [1]. Currently, surgery associated or not with specific antibiotic therapy is the only proven effective treatment of BU. However, this treatment can only be used in a few medical centers with proper and adequate equipment and is neither affordable nor accessible to an important part of the population [2]. So, in Benin, traditional treatment remains the first option for patients with generally low income resources [2, 3]. This treatment, in spite of its importance for patients is not adequately documented. Earlier studies carried out in Benin described some aspects of the use of traditional treatment in the population such as the cultural context and the different forms being applied [3, 4]. Most of the components in the traditional treatment belong to the plant kingdom, a fact that has not yet been mentioned in previous studies.

Recently we carried out an ethnobotanical survey involving seventeen

traditional practitioners within the Ouinhi community in the Zou department. We noted that forty nine different plants were used for the traditional treatment of BU. Different parts of these plants were used in various pharmaceutical forms for internal or external usage [5]. The chemical study of these 49 identified plants revealed the presence of alkaloids, tannins, flavonoids, saponins, quinonic derivatives, essential oils, steroids and terpenoids. Many chemical compounds of these groups are known for their antibacterial properties [5].

The aim of the present study is to investigate the antimycobacterial activity of traditionally used plant species on *M. ulcerans* and further to isolate and identify the active components in these species. Many researchers used the 96-well microplate format to screen substances for antimycobacterial activity against *M. tuberculosis*, using the alamar blue assay [6] or the resazurin assay [7,8,9] and most recently, to assess the antimycobacterial activity of several plant extracts, modified antibiotic molecules and antifungal Azole Drugs [10,11,12,13,14, 15, 16]. In this study the antimycobacterial bioassay was performed using the Resazurin Microtiter Assay (REMA) allowing the detection of microbial growth in a small volume of solution in microtiter plates.

2. Methodology

Plant materials
Out of 49 identified plants in Benin, 44 were used in this study. Names and used parts of studied plants are given in Table. The plants were identified by a botanist from the National Herbarium of Benin and voucher specimens are deposited at the same herbarium.

Preparation of extracts and fractions
Dried plants were ground to a powder with a pulverizator (National Mixer Grinder Mx-119N, Japan). 50g of powder was then macerated 48h 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 40°C using a Rotavapor (Buchi Rotavapor R-200/205, Switzerland) to obtain a crude residue. Then, the residue was lyophilised (Labconco Freeze Dryer 4.5, Belgium) and was kept in the dark at 4°C until use.

Solutions of plant extracts
The solutions of test plant extracts were prepared by dissolving the extracts in 100% dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) resulting in a stock solution of 40mg/ml; and then further diluted with sterile distilled water and 7H9 Middlebrook medium (Middlebrook 7H9 supplemented with 0.1% casitone, 0.5% glycerol, and 10% OADC [oleic acid, albumin, dextrose, and catalase] Becton-Dickinson, USA) to obtain a final working dilution of 1mg/ml. Solutions were filter sterilized In order to be sure that there were no inhibitory effects on the growth of *M. ulcerans* a control experiment was performed with DMSO.

Redox-indicator resazurin
Resazurin sodium salt powder (Acros Organic N.V., Geel, Belgium) was prepared at 0.02% (w/v) in distilled water, filter sterilized, and stored at 4°C for no more than 2 weeks.

Antimycobacterial resazurin assay
We determined the MIC using the resazurin assay (REMA) according to Palomino et *al*. [8] with small modifications in the procedure. These modifications concerned Mycobacterium strain, a concentration of mycobacterial suspension and conditions of Incubation.

M. ulcerans reference strain ATCC 19423 from the mycobacterial collection of the Institute of Tropical Medicine, Antwerp, Belgium, was used. Fresh colonies of *M. ulcerans* from Löwenstein-Jensen medium were suspended in Middlebrook 7H9-S broth at a concentration of 1 mg/ml. A total of 100 µl of this suspension was added to each well of a microtiter plate together with the plant extracts in Middlebrook 7H9-S broth to obtain a final volume of 200 µl in each well. The final concentration of the plant extracts were 250, 125, 62.50, 31.25, 15.62, 7.81 µg/ml respectively. We included 3 positive control wells (containing 100 µl of Middlebrook 7H9-S broth and 100 µl of mycobacterial suspension each) and 3 negative control wells (containing 200 µl of Middlebrook 7H9-S broth). Our reference drug was rifampicin. After incubation of 15 days, 30 µl of resazurin 0.02% were added to the first positive control well. If the dye turned pink, indicating bacterial growth, the dye was then added to all remaining wells in the plate.

The results were read 48 hours later and reported. If we recorded no color change until the 17th day (thus 2 days after the addition), results were considered inconclusive. On the other hand, if the dye turned to pink after the addition, we would only consider the results if and only if the negative control wells colored blue on addition of resazurin.

The MIC (Minimal Inhibitory Concentration) is defined as the lowest concentration of extract that prevents a color change of resazurin (blue to pink). The plant extracts that could not prevent growth of *M. ulcerans* up to a concentration of 250 µg/ml are considered inactive [17].

3. Result

Out of 44 plant extracts tested, two inhibited the growth of *M. ulcerans: Jatropha curcas* at a concentration of 250 µg/ml and *Holarrhena floribunda* at 125 µg/ml (see table I). Control experiments showed that 0.625 % DMSO or less in each well did not have any inhibitory effect on the growth of *Mycobacterium ulcerans* ATCC 19423.

4. Discussion

In traditional medicine, practitioners use different plant combinations to treat BU, but in our study plants were investigated individually. This could explain the low number of plants found to be active against *M. ulcerans*. It is interesting to highlight the fact that there were a number of plants, reportedly used in traditional medicine to treat BU in Benin, which did not demonstrate any antimycobacterial activity *in vitro*. It is probable that these plants are used to treat the symptoms of the disease rather than actually to kill the bacteria. Some plant species may not contain compounds which inhibit the growth of or kill *M. ulcerans* but may have anti-inflammatory, analgesic, anaesthetic, antiseptic, anti oedema, or healing properties. It could have been of benefit if this study is followed with the combination effects of some of the other plant species and find out if any combinations had any synergistic effect. This could be done if practitioners accept to give their remedies instead of keeping it like secret.

For screening antibacterial activities of natural products, as crude extracts, it is essential to use an *in vitro* antibacterial assay that is simple, rapid, sensitive, and cost-effective. Usually, small quantities of natural products are available for antibacterial screening and this can be a limiting factor. The conventional method performed on Löwensten Jensen medium is time consuming, very slow, especially when testing *M. ulcerans* and requires significant quantities of materials. We therefore decided to focus on the REMA test to screen plant extracts for antimycobacterial activity against *M. ulcerans*. The resazurin assay using a microtiter plate, described here has been modified to determine the MIC values of natural products against M. ulcerans. Control experiments showed that 0.625 % DMSO or less in each well did not have any inhibitory effect on the growth of *Mycobacterium ulcerans* ATCC 19423. The REMA method is simple and rapid and could be used successfully to assess antibacterial properties of natural products. The 96 well microplates offer the advantage of using small volume of reagents, it is low cost method and the plate can be read visually without the need of instrumentation. It was used to screen plant extracts and allowed us to select two extracts with promising activity against *M. ulcerans*, which may lead to the discovery of new antimycobacterial compounds..

Table: Plant materials used for antimycobacterial activity screening and MIC values of their ethanol extracts.

Voucher specimen	Family name	Scientific name	Plant parts	MIC (µg/l)
Yemoa1	Anarcadiaceae	*Lannea kerstingii Engl. et K. Krause*	leaves	>250
Yemoa2	Anarcadiaceae	*Spondias mombin Linn*	stem bark	> 250
Yemoa3	Annonaceae	*Xylopia aethiopica (Dunal) A. Rich*	fruit	>250
Yemoa4	Annonaceae	*Monodora myristica (Gaertn) Dunal*	seeds	>250
Yemoa5	Apocynaceae	*Strophanthus hispidus DC*	Root	>250
Yemoa6	**Apocynaceae**	***Holarrhena floribunda (G.Don) T.Durand et Schinz*[(a)]**	**leaves**	**125**
Yemoa7	Araceae	*Anchomanes difformis Engl*	rhizome	> 250
Yemoa8	Asteraceae	*Launaea taraxacifolia (Wild.) Schum*	leaves	>250
Yemoa9	Asteraceae	*Vernonia amygdalina Del*	leaves	>250
Yemoa10	Bignoniaceae	*Spathodea campanulata (P.Beauv)*	stem bark	> 250
Yemoa11	Bignoniaceae	*Stereospermmum kunthianum (Cham)*	root	>250
Yemoa12	Bignoniaceae	*Newbouldia laevis (P.Beauv) Seeman*	Root	>250
Yemoa13	Caesalpinaceae	*Erythrophleum suaveolens (Guill et Perr.) Brenan*	Stem bark	> 250
Yemoa14	Caesalpinaceae	*Piliostigma thonningii (K.Schum.) Milne-Redh*	leaves	> 250
Yemoa15	Capparaceae	*Ritchiea capparoides (Andrews) Britten*	root	> 250
Yemoa16	Chenopodiaceae	*Chenopodium ambrosioides Linn*	leaves	> 250
Yemoa17	Clusiaceae	*Garcinia kola Heckel*	root	> 250
Yemoa18	Combretaceae	*Anogeissus leiocarpus (DC.) Guill et Perr*	leaves	> 250
Yemoa19	Combretaceae	*Terminalia glaucescens Planch*	bark / root	> 250
Yemoa20	Crassulaceae	*Bryophyllum pinnatum (Lam.) Okem*	leaves	>250
Yemoa21	Cucurbitaceae	*Kedrostis foedissima (jacq.) Cogn.*	leaves	>250
Yemoa22	Euphorbiaceae	*Euphorbia kamerunica Pax*	bark	>250
Yemoa23	Euphorbiaceae	*Hymenocardia acida Tul*	bark	>250
Yemoa24	Euphorbiaceae	*Bridellia ferruginea Benth*	bark	>250
Yemoa26	**Euphorbiaceae**	***Jatropha curcas Linn*[(a)]**	**leaves**	**250**
Yemoa27	Euphorbiaceae	*Jatropha gossypifolia Linn*	leaves	>250
Yemoa28	Fabaceae	*Lonchocarpus cyanescens (Schumch et Thonn) Benth*	root	>250
Yemoa29	Lamiaceae	*Ocimum gratissimum Linn*	leaves	>250
Yemoa30	Lamiaceae	*Ocimum canum Sims*	leaves	>250
Yemoa31	Liliaceae	*Allium cepa Linn*	bulb	>250
Yemoa32	Liliaceae	*Aloë buettneri A. Berger*	leaves	>250
Yemoa33	Melastomataceae	*Dissotis rotundifolia (Sm.) Triana*	Leaves	>250
Yemoa34	Mimosaceae	*Tetrapleura tetraptera (Schum. et Thonn) taub.*	fruit	>250
Yemoa35	Moraceae	*Ficus exasperata Vahl.*	leaves	>250
Yemoa37	Myrtaceae	*Eugenia aromatica (Linn.) Baill*	fruit	>250
Yemoa38	Nyctagynaceae	*Boerrhavia erecta Linn*	leaves	>250
Yemoa39	Periplocaceae	*Parquetina nigrescens (Afzel.) Bullock)*	leaves	>250
Yemoa40	Piperaceae	*Piper guineense (Schum. Et Thonn.)*	seeds	>250
Yemoa41	Poaceae	*Eleusine indica Linn*	plant	>250
Yemoa44	Rutaceae	*Clausena anisata (Wild.) Hook.f*	root	>250
Yemoa45	Sapindaceae	*Paullinia pinnata Linn*	leaves	>250
Yemoa46	Sapotaceae	*Vitellaria paradoxa Gaertner*	leaves	>250
Yemoa48	Zingiberaceae	*Aframomum melegueta K. Schum*	fruit	>250
Yemoa49	Zingiberaceae	*Curcuma longa L.*	leaves	>250

(a) plantes actives

5. Conclusion

The REMA method is simple and rapid and could be used successfully to investigate antimycobacterial activities of natural products against this microorganism responsible for BU, an important emerging disease. This could stimulate interest in research of active natural products against *M. ulcerans*. Phytomedicine is the first treatment used by the poor local population. Additional studies are however required to investigate the possible efficacy of some natural products for the treatment of BU when the WHO recommended treatment cannot be applied immediately.

Among plants tested, two showed promising activity: *Jatropha curcas* and *Holarrhena floribunda*. It is clear that more research is needed to isolate and identify the active compounds of these plant extracts. Fractions of the plant extracts that showed in this study inhibiting activities are under investigation in our laboratory for further studies.

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Objectifs

1. Promouvoir une recherche pour l'action et le changement dans le domaine de la santé au sein de la CEDEAO.
2. Créer un environnement favorable à la dissémination de l'information sur la recherche en santé
3. Assurer le plaidoyer en vue de mobiliser des ressources et la disponibilité des produits de la recherche
4. Soutenir la formation et le renforcement des capacités des institutions de recherche et des chercheurs
5. Disséminer /diffuser les informations sur les activités de recherche dans l'espace CEDEAO.
6. Promouvoir le partenariat durable entre les chercheurs et les parties concernées par la recherche dans l'espace CEDEAO

Objectives

1. Promote quality research for action and change in the area of health within the ECOWAS area.
2. Create an enabling environment for dissemination of information on research for health
3. Assure the advocacy to mobilize resources and the availability of research products
4. Facilitate the capacity building of research institutions and researchers
5. Disseminate / spread information on research activities within the ECOWAS area.
6. Promote partnership between researchers and concerned parties in research inside and outside the ECOWAS area

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