

Antimycobacterial Effects of Herbal Preparations Used In Buruli Ulcer Endemic Communities In Ghana

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Research Article

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Abstract

Buruli ulcer is a devastating skin infection caused by *Mycobacterium ulcerans*. It is endemic in the Amansie West district of the Ashanti region of Ghana where patients rely mainly on herbal preparations from practitioners. This work aimed at validating some preparations employed by traditional healers in the Amansie West district for the management of Buruli ulcer. Four herbal preparations, obtained from practitioners in four communities were tested against the clinical *M. ulcerans* strain (NM203) using the resazurin microplate assay. Cytotoxicity test was done on the extracts using Chang liver cells. Chemical profiling of the active formulation was done using High-performance liquid chromatography. Some traditional herbal practitioners considered painless wounds in the extremities as diagnostic of BU whereas others relied on diagnosis of BU from a recognized conventional healthcare facility before proceeding to treat with herbal products. A formulation containing the roots of *Cryptolepis sanguinolenta* and seeds of *Picralima nitida*, as plant ingredients, was considerably active (MIC = 32µg/mL). The preparations were non-cytotoxic to Chang- liver cells. The herbal preparations showed considerable antimycobacterium ulcerans activity and are not toxic. A major peak in the HPLC chromatogram of the active formulation with a retention time of 17.86 min was identified as a marker for the quality analytics of the active formulation. A traditional herbal preparation used in Buruli ulcer endemic community was validated to show antimycobacterium ulcerans activity. Validation of similar preparations could afford novel drugs or products for this neglected tropical disease.

1. Introduction

Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is an indolent necrotizing disease of the skin, subcutaneous tissue, and bone. The disease is presently the third most common mycobacterial disease of humans, after tuberculosis and leprosy (Sizaire et al. 2006). BU is endemic in rural wetlands of tropical countries of Africa, the Americas, Asia, and Australia but remains uncommon in non-African countries. Incidence rate of the disease is currently highest in West Africa, particularly Benin, Côte d'Ivoire, and Ghana (Portaels et al. 2009).

A 1999 active case search resulted in a prevalence rate of 20.7 per 100,000. The Amansie West district, drained by the Offin river, was identified as the most endemic (Amofah et al. 2002). A case search along 13 communities of the river revealed an overall prevalence of 2.3 % (Ampah et al. 2016). The perception that the disease is caused by witchcraft and curses, the reluctance of patients to seek treatment outside their own communities (Stienstra et al. 2002), and the shortcomings of the present drug treatment of Rifampicin- Streptomycin, coupled with the strong cultural acceptance of traditional medicine makes the adoption of herbal medicines, in these communities an easier and preferred option. In this context, among the low-to-middle income countries (LMICs) in Africa, particularly Ghana, there is a sustained interest in the use of natural products (NPs) including medicinal plants to provide new drugs for BU.

There exist reports of ethnobotanical studies, which have identified several individual medicinal plants used in the management of BU across Africa (Balde et al. 2015; Bayaga et al. 2017; Fokou et al. 2015;

Gbekley et al. 2020; Liwata et al. 2020; Yemoa et al. 2008; Zabo et al. 2019). Some have gone ahead to validate the *in vitro* antimycobacterial effects of these plant extracts and some of their chemical constituents (Donfack et al. 2014; Fokou et al. 2016; Tsouh Fokou et al. 2016; Yemoa et al. 2015). Further to these, several earlier reports have highlighted the knowledge, attitude and practices of patients, community leaders and household heads of Buruli ulcer endemic communities (Akoachere et al. 2016; Renzaho et al. 2007). However, no efforts at understanding the knowledge, attitudes and practices of traditional healers involved in BU treatment in Ghana is on record.

Buruli ulcer may induce severe disabilities impacting on a person's well-being and quality of life. Even worse is the participation restrictions experienced by former patients, with most of these related to employment (de Zeeuw et al. 2014). For these patients the continued search for Buruli ulcer remedies and the scientific validation of these products remain relevant. Validation of traditional medicinal products have yielded some clinically useful drugs (Fokunang et al. 2011). An ethnopharmacological study of herbal formulations used in the management of BU, in the endemic communities in Ghana, is thus relevant as traditional medicine continues to play a key role in the maintenance of health and prevention of disease in these communities as well as drug discovery (Fokou et al. 2015).

Consequently, this work is aimed at identifying the diagnostic tools, treatment goals, preparations employed by traditional healers in the Amansie West district of Ghana. Secondly, the study is targeted at collecting some herbal formulations from the practitioners and evaluating their constituent plants for anti-mycobacterium activities in *in vitro* studies.

2. Materials And Methods

2.1 Ethnobotanical survey

In-depth interviews were conducted for the THs to assess how they make a diagnosis of Buruli ulcer, what treatments they administer and the duration of the treatments, how the treatments are prepared and what the treatment endpoints are? Qualitative data was tape-recorded and manually transcribed, coded according to responses of respondents after obtaining an oral consent.

2.2 Study location

The study was conducted in the Amansie West district of the Ashanti region, the most endemic district in Ghana which lies in the tropical rainforest belt with a large farming population (Fig. 1). It has a vegetation mix of virgin and secondary forest and is drained by the Offin River. The 2012 prevalence rate of 151 cases per 100,000 inhabitants exceeded the national rate of 22.7 cases per every 100,000 inhabitants. The district has a specialized Buruli ulcer (BU) treatment hospital at Agroyesum, where management of the disease is strictly according to WHO guidelines. Herbal formulations used in the management of Buruli ulcer were obtained from herbal practitioners in four endemic communities of the Amansie West district namely, Agroyesum, Bonsaso, Keniago, and Tontokrom.

2.3 Study population and data collection

The target group in this study were herbalists who had claims of successfully treating the infections/ulcers with their preparations. They were enrolled for the study after they consented. Information was obtained using interview guides designed to collect data on the diagnosis of BU, herbal preparations employed, how they are administered. Samples of the herbal preparations used were collected and subsequently subjected to *in vitro* antimycobacterial assays.

In another follow up interview, the findings from the initial antimycobacterial screens were shared with the traditional healers. Information on the individual plant constituents of the preparations were obtained.

2.4 Instruments

Chromatographic fingerprint was obtained on a PerkinElmer Flexar HPLC system consisting of a binary LC pump, LC autosampler, PDA plus detector, in-line degasser equipped with Chromera software. Synergy H1 Hybrid reader from Biotek was used for microplate readings.

2.5 Collection and extraction of herbal formulations

Four powdered herbal formulations were obtained from each of the endemic communities in September 2017 and coded formulations 1–4 (Table 1). The methods employed in the extractions were done to mimic closely what the traditional healers recommended to their clients (Table 1). About 200 g each of the herbal preparations were weighed. Two of the formulations, F1 and F2, were macerated in ethanol (96%), filtered and evaporated to dryness using a rotary evaporator. The other two, F3 and F4, were cold macerated overnight in water, filtered and the marc subjected to further decoction for 40 min and filtered. The aqueous extracts were pooled and lyophilized. The dried extracts were kept in glass vials and kept in the refrigerator until required for use.

2.6 Chemical profiling

2.6.1 Chromatographic fingerprinting

Fifty milligrams of Formulation 3 were dissolved in 10 ml of water and injected onto the flow stream of the mobile phase with an injection volume of 20 μ l. The separations were developed on a 3.9 x 300 mm Phenomenex C18, 5 μ m column using a flow rate of 1 ml/min and a mobile phase of H₂O and MeOH with a linear gradient from 1–100% MeOH in 40 minutes. A flow rate of 1 ml/min was used, and UV detection was performed at 274 nm.

2.7 Bioassays

2.7.1 In-vitro anti-mycobacterial assay

M. ulcerans clinical isolates (NM203), obtained from the Department of Bacteriology NMIMR, were used in this assay. The *M. ulcerans* NM203 were cultivated using a method previously described by (Fokou et al. 2016).

The mycobacterial inoculum was prepared by emulsifying a loop full in sterile Middlebrook 7H9 broth supplemented with 0.2% glycerol (Sigma-Aldrich, Steinheim, Germany) (7H9-GC) and adjusted to 1×10^6 cells/mL. The cultures were standardized to 1 McFarland concentration.

2.7.2 Drug susceptibility assay

Extracts were evaluated for antimycobacterial effects using the Resazurin microtiter assay (Yemoa et al. 2015) in round bottom 96-well plates. A stock extract concentration of 102.4 mg/mL was prepared in DMSO (100%). Ten microliters of this stock solution were pipetted into 990 μ L 7H9-G medium supplemented with 10% OADC (oleic acid-dextrose-catalase). Two hundred microliters of the resulting concentration (1024 μ g/mL) of this stock solution were pipetted into the first well of microtiter plate. Dilutions were made from the first concentration by two-fold serial dilutions in 7H9-G medium. The resultant concentrations in the wells were between 1000–32 μ g/mL. Tests were performed in triplicates. Rifampicin and streptomycin were used as reference drugs at highest concentrations of 4 and 8 μ g/mL respectively and serially diluted (two-fold) to obtain six concentrations. Wells containing the medium and viable *M. ulcerans* were used as negative control. Additional control wells had only the culture medium. The plates were stored in an incubator at 35°C for 15 days. Twenty microliters (20 μ L) of resazurin (0.01 %w/v) was added to the wells after 12 days of incubation just to ensure the organisms were viable. The plates were incubated for the next 2 days. The change of blue color of resazurin to red resorufin in the negative control well is considered positive for bacterial viability. The lowest concentration of extract under test that was able to inhibit the growth of organism was considered as the MIC.

2.7.3 Cytotoxicity assay

Chang liver (Cell line from human hepatocytes) were obtained from the Department of Clinical Pathology Department (NMIMR) and the cytotoxicity evaluated according to standard modified guidelines adapted from (Borra et al., 2009). From sub-confluent cultures in 75 cm² culture flasks, the Chang liver cells were trypsinized, counted and suspended in RPMI containing 10% Fetal bovine serum and 1% penicillin-streptomycin-glutamine (Sigma, Ayrshire, UK) and then were seeded into triplicate wells of a 96-well plate (100 μ L per well) at concentrations of 1×10^5 cells/mL and incubated at standard culture conditions of 5% CO₂ in air at 37°C. Cells were then allowed to attach overnight (a check for viability) and later treated with 10 μ L product extracts at concentrations of 62.5–1000 μ g/mL and incubated for 48 h. After incubation, 20 μ L of 0.15 mg/mL resazurin solution was added to each well and incubated for 4 h. The optical densities of the solution were recorded with the aid of spectrophotometer 570 nm. Control and test drug were assayed in triplicate with the results expressed as the mean $\pm$ SD. The positive control drug used in the assay was Curcumin at a concentration of 18 μ g/mL. The blue colour of the resazurin dye in the presence of a viable cell changed colour to a red resorufin dye which can be used as an indicator of

cytotoxicity. Cell viability was compared with the untreated cells. The value of the data was normalized as a percentage of cell activity. The concentration of the crude drug that can inhibit 50% of the proliferative activity of the cell is calculated as the IC₅₀. IC₅₀ was calculated by plotting the percentage viability of cells against the log concentration of the extracts.

3. Results

3.1 Herbal formulations sampled from endemic areas

Four (4) traditional healers residing in 4 communities in the Amansie West district were interviewed. All the interviews were conducted at the practicing site of the TH. Descriptions together with interview quotes that emphasize the descriptions are provided here.

Buruli ulcer was endemic in the communities the THs practiced. The THs were dependent on the physical observations the patients presented to confirm a case of BU. Others were dependent on the detailed diagnosis made at the BU hospital at Agroyesum.

"I usually consider any wound on the skin, especially in the lower and upper limbs, which is painless to touch as a sure bet for BU." TH1
"Most of the patients I treat are patients who have been diagnosed as having BU at the Agroyesum Hospital" TH2

Four formulations used to treat Buruli ulcer were listed (Table 1). The traditional remedies were applied as poultices or as a decoction with the respective routes of administration been dermal and oral. These formulations are administered for a couple of weeks or until the ulcerations are healed.

"I apply the decoction or poultice of the powdered polyherbal product both orally and topically until the ulceration is healed." TH3

Table 1. Herbal formulations used by Traditional Healers in Amansie West District for the treatment of Buruli Ulcer

ID of herbalist	Community	Herbal material	Medicinal plant ingredients	How is the preparation made and applied	Mode of application
Amansie 1	Keniago	Formulation 1	Stem bark of <i>Spathodea campanulata</i> P.Beauv. (Bignoniaceae) and roots of <i>Jathropa curcas</i> L. (Euphorbiaceae)	Bitters Powder	Topical Poultice
Amansie 2	Bonsaso	Formulation 2	Roots of <i>Ricinus communis</i> L. (Euphorbiaceae) and stem bark of <i>Khaya senegalensis</i> (Desv.) A. Juss. (Meliaceae)	Bitters Powder	Topical Poultice
Amansie 3	Agroyesum	Formulation 3	Dried roots of <i>Cryptolepis sanguinolenta</i> (Lindl.) Schltr. (Apocynaceae) and seeds of <i>Picralima nitida</i> (Stapf) T.Durand & H.Durand (Apocynaceae)	Decoction Or Powder	Topical Poultice
Amansie 4	Tontokrom	Formulation 4	Stem barks of <i>Bombax buonopozense</i> P.Beauv. (Malvaceae) and <i>Newbouldia laevis</i> (P.Beauv) Seem. Ex Bureau (Bignoniaceae)	Decoction	Oral

3.2 CHEMICAL PROFILE

The HPLC fingerprint of the most active extract was collected (Fig. 2). The chromatogram is a fingerprint of the formulation and can be used to preliminary establish quality of the formulation and subsequently its antimycobacterial effects.

HPLC chromatogram of formulation 3 could be used in the quality analysis of the product. The compound eluting at a retention time of 17.86 min, representing 35.49% (based on peak areas) of the constituents of F3, could be considered an important marker for the quality analytics of this product.

3.3 Antimycobacterial Activity

In the antimycobacterial activity, the four herbal formulations collected from the practitioners in the Amansie West district of the Ashanti region of Ghana were evaluated *in-vitro* against clinical isolates of *M. ulcerans* (NM203). Formulations 2 and 4, did not show any considerable activity as the MIC's were greater than 512 µg/mL (Table 2). Formulations 1 and 3 displayed considerable activities against the

clinical isolates (NM203). The reference drugs streptomycin and rifampicin, however, displayed excellent activities against the *M. ulcerans* organism with respective MICs of 1 and 0.5.

In the cytotoxicity assays, all four formulations were not cytotoxic at the highest concentration of 1000 µg/mL against Chang liver (Table 2).

Table 2
Anti-mycobacterium ulcerans and cytotoxic activities of
herbal formulations

Herbal Formulation	MIC(µg/mL)	Cytotoxicity IC ₅₀ /(µg/mL)
Formulation 1	125	> 1000
Formulation 2	> 512	> 1000
Formulation 3	32	> 1000
Formulation 4	> 512	> 1000
Rifampicin	0.5	> 1000
Streptomycin	1	> 1000
Curcumin		6.9

Discussion

The interviews with the THs revealed that herbal preparations were a key component for the management of BU patients, either diagnosed at the BU clinic or by the presence of painless-to-touch wounds on the limbs. Altogether, eight plants belonging to five families were identified (Table 1). *C. sanguinolenta*, *S. campanulata*, *N. laevis*, *J. curcas* and *R. communis* had been previously reported to have ethnomedicinal use in the management of BU (Yemoa et al. 2011; Tsouh Fokou et al. 2016; Amponsah et al. 2021). Despite their reported uses in skin disorders, including eczema and abscesses, *P. nitida*, *K. senegalensis* and *B. buonopozense* are mentioned here for the first time for their use in the management of BU. These three plants could be investigated individually for their anti-mycobacterial effects.

The anti-mycobacterial effects of the four extracts/formulations, ranged from 32 to > 512 µg/mL. The study demonstrated that formulation, F3 had the highest anti-mycobacterial activities against the clinical strain of *M. ulcerans* NM203. Of the stated constituent plants of F1, *C. sanguinolenta* and cryptolepine, its major alkaloid, had been shown to possess inhibitory effects on the clinical strain NM203 (Amponsah et al. 2021) and may be responsible, in part, for the observed effects. However, *P. nitida* seeds included in the formulation have not been previously investigated for their antimycobacterial effects. Formulation F1, containing stem bark of *S. campanulata* and roots of *J. curcas*, also showed inhibitory effects on *M.*

ulcerans. Both these plants had been previously investigated and established to have antimycobacterial effects (Yemoa et al. 2011; Tsouh Fokou et al. 2016).

According to the established criterion (Yemoa et al. 2011), MIC < 250 µg/mL, for submitting crude extracts for further investigations, formulations F2 and F4, were considered to have weak antimycobacterial effects. This notwithstanding, the individual plants could be subjected to bioassay-guided fractionation to identify their anti-mycobacterial compounds. The increasing reports of anti-TB drug resistance coupled with the continued BU infections present in sub-Saharan Africa, make the search for new natural products for BU more imperative.

At the highest concentrations, no cytotoxic effects were observed for the extracts, against the Chang liver cells. Chang liver cells provide an acceptable model to explore general cellular cytotoxicity as it possesses characteristics associated with hepatocyte function (Oteng et al. 2014). Thus, the four herbal formulations used by THs in the Amansie district are non-toxic and do not represent a present danger to patients. However, there is a need to corroborate these findings with toxicity data of the formulations from animal studies. Our inability to explore the wound healing activity of the formulations is the limitation of the current studies.

Conclusion

Our findings indicate that herbal formulations used in BU treatment possess antimycobacterial and non-cytotoxic properties in *in vitro* assays. These findings support the use of some mentioned medicinal plants in polyherbal formulations for the management of Buruli ulcer. in the Amansie West district.

Declarations

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ETHICS STATEMENT

Informed consent was obtained from the traditional healers (THs) prior to recruitment into the study. Only willing individuals were recruited for the study, and they were provided with all the necessary information regarding the study and their roles.

COMPETING INTEREST

The authors declare no competing interest.

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Figures

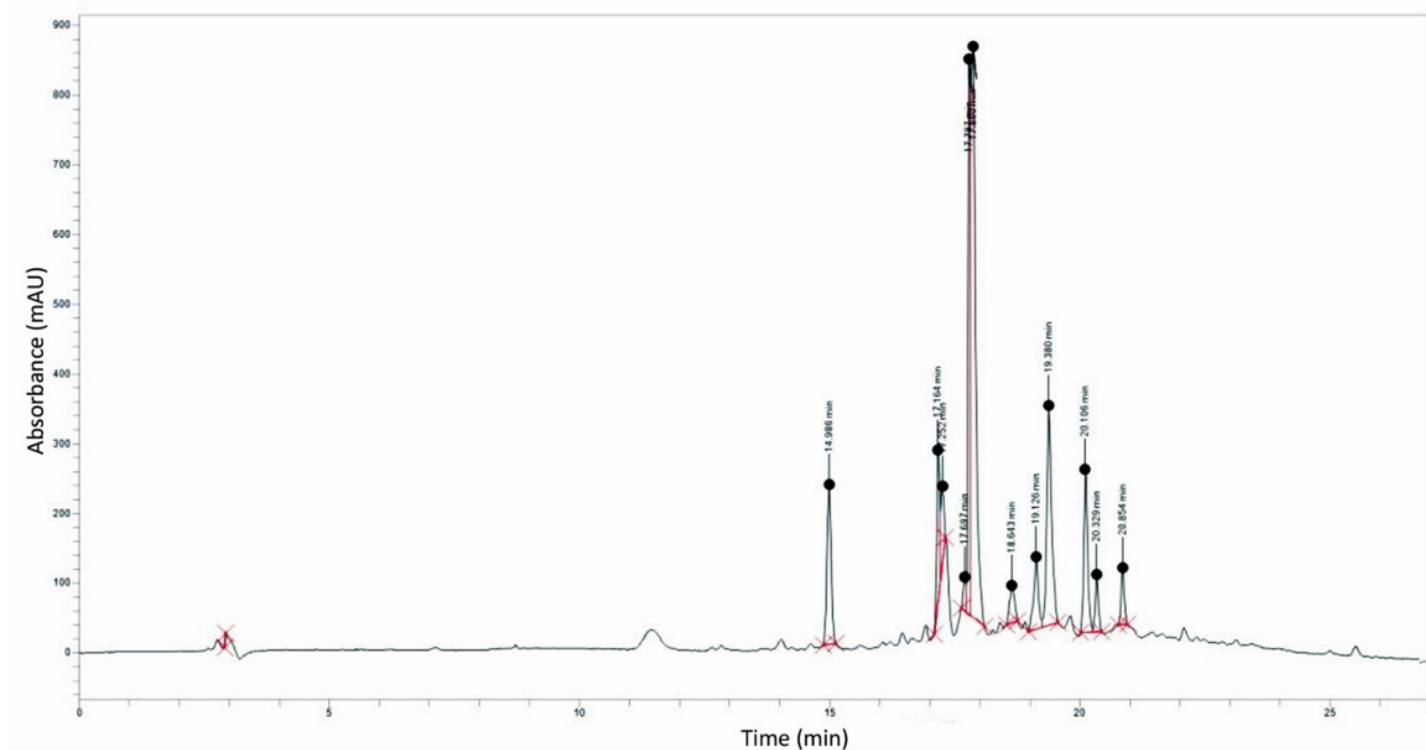


Figure 2

HPLC chromatographic fingerprints of the aqueous extract of Formulation 3.