In-Vitro Susceptibility of Mycobacterium Ulcerans to Herbal Preparations

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Abstract

Buruli ulcer (BU) is a skin disease caused by Mycobacterium ulcerans. Surgery is the main treatment option because antibiotics have mostly been ineffective. However, there are reports of successful treatment with undisclosed herbal preparations. This study screened 44 herbal preparations for (i) inhibitory activity against 7 M. ulcerans isolates, (ii) determined the minimum inhibitory concentrations (MICs) of 10 herbal preparations and (iii) screened them for extended antimicrobial activity, since BU is often associated with secondary bacterial infections. Twenty-five of the herbal preparations inhibited the growth of the M. ulcerans isolates. MICs of the 10 herbal preparations were between 0.20 and 12.50% volume/volume (1:128 - 1:2), and all 10 demonstrated extended antimicrobial activity. The findings (i) indirectly confirmed the claims that BU is being successfully treated with herbal preparations and (ii) suggest that the treatment of BU with herbal preparations might simultaneously treat secondary bacterial and fungal infections. In conclusion, there are a number of herbal preparations with anti-M. ulcerans activity, therefore there is the need to pay more attention to herbal therapy as a potential BU treatment option.

INTRODUCTION

Buruli ulcer (BU) is a re-emerging disease caused by Mycobacterium ulcerans, a toxin-producing bacterium with predilection for the skin and its deeper tissues. The exact mode of its transmission is unclear, and the indolent progression of the disease often results in massive ulceration of the skin with impairment of body functions. BU has been reported in many tropical countries in North and South America, Southeast Asia, Australia and Africa (Portaels et al., 1998; WHO, 2001), where it is endemic and its prevalence is higher than that of tuberculosis and leprosy in some communities (Amofa et al., 2002).

Clinically, BU could present on any part of the body as a papule, nodule, plaque, oedema or ulcer with deeply undermined edges (WHO, 2001; Johnson et al, 2005); some of the skin conditions become cancerous with time (WHO, 2001). BU is often secondarily infected by bacteria and fungi (those often implicated include Staphylococcus aureus, Staphylococcus sp., Streptococcus sp. Pseudomonas sp and yeast cells) (WHO, 2001), which sometimes results in septicaemia, osteomyelitis and cellulitis (WHO, 2001). The treatment of BU with antimicrobial agents has presented varying degrees of success, leaving surgery as the main treatment option, which also accounts for a large proportion of surgical bed occupancy (Thangaraj et al., 1999) and unaffordable hospital bills for the rural poor, who are incidentally, those mostly affected by the disease) (Amofa et al 2002). Though recent data suggests that combinations of antimycobacterials that include rifampicin, streptomycin or amikacin are effective in combination with surgery (WHO, 2003; WHO, 2004; Etuaful et al., 2005), recurrences still occur and advanced ulcers are still difficult to treat (Teelken et al., 2003; Etuaful et al., 2005). In view of this, the search for an effective antimycobacterial that can substitute surgery, prevent the dissemination, recurrence and complications associated with the disease, is a priority.

In Ghana, there are unsubstantiated claims of the successful treatment of BU patients by traditional herbal practitioners (THPs) with undisclosed herbal preparations. This study therefore primarily sought to identify herbal preparations with inhibitory activity against Mycobacterium ulcerans, from among herbal preparations purportedly used in Ghana for the treatment of sores, wounds, skin disorders, swellings, gastric and diabetic ulcers, cancers, infectious diseases, and

in some instances even Buruli ulcer. The selection of the herbs and herbal preparations was on the premise that since Mycobacterium ulcerans has predilection for the skin and its deeper tissues, BU may be effectively controlled with herbal preparations employed in the treatment of skin conditions (of infectious and non-infectious origin) and internal ulcers. The study also set out to determine the minimum inhibitory concentrations (MICs) of the effective herbal preparations and finally screen them for extended antimicrobial activity, in view of the secondary infection of BU with fast-growing microorganisms (WHO, 2001).

MATERIALS AND METHODS

BACTERIAL STRAIN

Seven Mycobacterium ulcerans strains were investigated. Five were obtained from biopsies of BU patients from two BU endemic areas (Amasaman, Nsawam) in Ghana, while 2 were American Type Culture Control (ATCC) reference isolates (Ghana #970321 (D19F9), Benin #990826 (D27D14)) provided by Professor Françoise Portaels of the Institute of Tropical Medicine, Antwerp, Belgium. Prior to their use, the 2 reference and 5 clinical isolates were confirmed for their authenticity and purity as M. ulcerans isolates by phenotypic tests (WHO, 2001) and polymerise chain reaction (PCR) (WHO, 2001).

MICROBIAL STRAINS OTHER THAN

Nineteen microorganisms (6 Gram-positive bacteria, 10 Gram-negative bacteria and 3 fungi) were used to investigate any extended antimicrobial property the herbal preparations may possess. They microbes consisted of 11 biochemically-authenticated clinical isolates (Stretococcus sp., Staphylococcus sp., Bacillus sp., Haemolytic E. coli, Klebsiella sp., Salmonella sp., Salmonella typhimurium, Pseudomonas aeruginosa, Shigella sonnei, Aspergillus sp., Candida albicans) and 8 reference isolates (Staphylococcus aureus – ATCC 6538; Bacillus cereus – VDL 173; E. coli – ATCC 8739; Pseudomonas sp.- ATCC 27853; Enterococcus faecalis – VDL 122; Salmonella adabraka – VDL 275; Bacillus subtilis – ATCC 6633 and Aspergillus niger – ATCC 16404)

MEDICINAL PLANTS AND HERBAL PREPARATIONS

Forty-four medicinal plants and herbal preparations (Table 1) were obtained for the study, based on their purported antimicrobial and anti-inflammatory properties, which are exploited for the treatment of Buruli ulcer, various skin conditions, sores, new and old wounds, gastric and diabetic ulcers and cancers. The herbal preparations were obtained from: (i) Traditional herbal practitioners, (ii) a vendor of traditional herbal medicines, (iii) 2 herbal shops and (iv) users of traditional herbal preparations. All the plant materials were authenticated at the Botany Department of the University of Ghana, with the exception of 4 imported herbal preparations. The 4 imported herbal preparations (Swedish bitters, Hydrastis canadensis, Chamomilla recutita and Symphytum officinale) have extensive patronage in Ghana. Swedish bitters is an already-bottled alcoholic extract of 11 herbs (Aloe, Myrrh, Saffron, Senna, Camphor, Rhubarb root, Zedvoary, Manna, Theriac venezian, Carline thistle root, Angelica root) produced in Germany for NATUREWORKS, a division of ABKIT, INC, New York, NY 10128; while Hydrastis canadensis (dried roots), Chamomilla recutita (dried flowers) and Symphytum officinal (dried roots) are imported as such from Germany and retailed by a certified Herbal shop (Herbal Options Ltd, Ghana).

ANTIBACTERIAL AND ANTIFUNGAL AGENTS

Pefloxacin mesylate dehydrate and miconazole were used as reference drugs for the Gram-positive bacteria /Gram-negative bacteria and fungi respectively. Pefloxacin mesylate dehydrate is an antibiotic produced by WORKHARDT LTD of India, while miconazole is an antifungal produced by JANSSEN-CILAG LTD of Portugal.

BIOSAFETY CONSIDERATIONS

The study was undertaken in a level 2 biosafety laboratory. Microbiological procedures such as subculturing, inoculum preparation, standardization of microbial isolates and the inoculation of media with microbial isolates were all conducted in a class IIA/B_3 biosafety cabinet, while the technical team observed all institutional (NMIMR) biosafety guidelines for personnel protection and that of the laboratory.

PREPARATION OF INOCULA

The M. ulcerans isolates were subcultured on Lowenstein-Jensen (L-J) slants at 32 C for eight weeks, during which period they were examined for growth and contamination. Each of the M. ulcerans subcultures was suspended in sterile distilled water and standardized by the McFarland nephelometric standard. Briefly, a drop of sterile distilled water was added to a test tube containing 15-20 sterilized glass beads to wet them. A loopful of M. ulcerans subculture was added to the beads and drops of sterile distilled water were added intermittently and vortexed to break up the M.

ulcerans colonies and to adjust the turbidity of the suspension to that of a No. 1 McFarland. Since M. ulcerans in culture is very waxy and not easily dispersible in water, all manipulations were done on ice. The No. 1 McFarland standardized M. ulcerans suspensions were serially diluted 10-fold to yield 10-2 and 10-4 suspensions. Smears of the resultant M. ulcerans suspensions were stained with Ziehl Neelsen (ZN) stain for detection of acid-fast bacilli and to check for microbiological purity. The serially diluted suspensions were later used for the susceptibility test.

PROCESSING OF HERBAL PREPARATIONS

The medicinal plants were prepared into juices, infusions or decoctions and incorporated into media as such, to simulate the state in which consumers would use them. Swedish bitters, being a liquid extract was incorporated into media as such; also simulating the state in which it would be used.

PREPARATION OF HERBAL JUICES

Fresh rhizomes, bulbs, thick leaves and plants containing gel were prepared into juices. Weighed amounts of each herb was washed in running water, rinsed with sterile distilled water and blended with sterile distilled water in a sterile Waring laboratory blender. The resultant 20% w/v juice was strained with a sterile tea strainer and filtered through a sterile Whatman No. 1 filter paper. The filtrate was kept at 4 C until use the following day.

PREPARATION OF HERBAL INFUSIONS

Weighed amounts of whole plants or leaves were washed under running water, rinsed with sterile distilled water, shredded into bits, macerated overnight in sterile distilled water and subsequently boiled in a covered glass jar for 5 minutes. The infusion was strained in a sterile tea strainer, while pressing down the leaves (to get as much of the aqueous component from the plant as possible). The resultant 20% w/v infusion was left to cool and filtered through a sterile Whatman No. 1 filter paper and kept at 4 C until use the following day.

PREPARATION OF HERBAL DECOCTIONS

Weighed amounts of stems, barks and roots were prepared as decoctions. The stems, barks and roots were scrubbed in running water, rinsed with sterile distilled water, air dried in a clean air rack and dry blended into powder. The powders were then macerated overnight in sterile distilled water and subsequently boiled in a covered glass jar for 20 minutes. Each decoction was strained in a sterile tea strainer, while pressing down the powder (to get as much of the aqueous component from the plant as possible). The resultant 20% w/v decoction was left to cool and filtered through a sterile Whatman No. 1 filter paper and kept at 4 C until use the following day.

SCREENING OF HERBAL PREPARATIONS FOR ANTI- ACTIVITY

The herbal preparations were screened for demonstration of anti-M. ulcerans activity. Briefly, the herbal preparations (infusions, decoctions and juices) were each incorporated at 1:5 dilution into L-J medium and inspissated as media slants, alongside herb-free L-J media, which served as controls. Triplicates each of the herb-incorporated and herb-free media were each inoculated with 100µl of 10-2 No. 1 McFarland standardized M. ulcerans (Ghana & Benin reference isolates and 3 and 2 clinical isolates from Amasaman and Nsawam respectively) and incubated at 32 C for 8 weeks. Non-inoculated triplicates of the herb-incorporated media and herb-free media were incubated alongside to serve as drug and L-J media controls respectively. The tubes were read after 4 and 8 weeks of incubation. Herbal preparations were defined as having anti-M. ulcerans activity if all triplicate tubes were without a single M. ulcerans colony after 8 weeks of incubation, while there was growth on herb-free L-J slants, but no growth on non-inoculated controls (positive and negative respectively). A test in which the triplicate tubes had growth on one or two tubes was repeated.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS OF HERBAL PREPARATIONS

The herbal preparations that exhibited anti- M. ulcerans activity were incorporated into L-J media at two-fold serial dilutions (1st to 8th dilution). Consequently, the infusions, decoctions and juices were incorporated into L-J media at final concentrations ranging from 25% vol/vol to 0.20% vol/vol. Controls were set as in the screening test. Triplicates of each dilution of the herb -incorporated media and triplicates of herb-free control media were inoculated with 100µl of 10-2 No. 1 McFarland standardized M. ulcerans (Ghana & Benin reference isolates and the 5 clinical isolates from Amasaman and Nsawam). Triplicate drug-free slants were also inoculated with 100µl of 10-4 M. ulcerans suspension (Ghana & Benin reference isolates and 3 and 2 clinical isolates from Amasaman and Nsawam respectively). Non-inoculated triplicates of the herb-incorporated media and herb-free media were incubated alongside the inoculated ones to serve as drug and L-J media controls respectively.

All the tubes were incubated at 32 C for 8 weeks and read after 4 and 8 weeks of incubation. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of herb that completely inhibited M. ulcerans growth after 8 weeks of incubation, while both the 10-2 and 10-4 positive controls had growth but the negative (i.e. non-inoculated) controls had no growth, thus confirming the absence of contamination.

SCREENING OF HERBAL PREPARATIONS FOR EXTENDED ANTIMICROBIAL ACTIVITY

In view of the secondary infections that sometimes accompany BU, the herbal preparations that exhibited anti-M. ulcerans activity were further investigated for demonstration of extended antimicrobial property against 19 microbes which have the potential to cause or complicate skin diseases and osteomyelitis. The herbal preparations were screened by the agar dilution method. Briefly, each herbal preparation was incorporated into 5% blood agar or appropriate selective medium (Desoxycholate Hydrogen Sulfide Lactose Agar, Mannitol salt agar, Pseudomonas selective medium, Bacillus cereus selective agar, Xylose Lysine deoxycholate and Sabouraud dextrose agar) at a 1:5 dilution and incubated for 24 hours to check for sterility, alongside controls (i.e. herb-free medium, reference drug-incorporated medium). A pure culture of each microbe was prepared as a 0.5 McFarland standardized inoculum in sterile distilled water. Triplicates of sterile herb-incorporated, sterile reference drug-incorporated and sterile herb/reference drug-free (controls) agar plates were each inoculated with a bacteriological loopful (4mm diameter) of the inoculum and incubated together with inoculum-free controls (further sterility check) at 37°C for 24 and 48 hours. All plates were checked for microbial growth after 24 and 48 hours of incubation. An herbal preparation or reference drug was categorised as having antimicrobial activity against a particular microbe if the herb-incorporated or reference drug-incorporated medium was without microbial growth after 24-48 hours of incubation, while the positive controls had confluent growth and the negative controls had no growth within the same incubation period.

STATISTICAL ANALYSES

The Statistical Package for the Social Sciences (SPSS), version 12.0.1 was used for data analysis. The case summaries procedure was used to determine the means, standard deviations, minimum, maximum and median of the minimum inhibitory concentrations of the herbal preparations and susceptibilities of the isolates. After conducting the above-mentioned determinations the data were screened for normality and homogeneity of variance. Most of the data violated the assumptions of the normal distribution and since they could not be acceptably transformed they were analysed with nonparametric tests: (1) The Kruskal-Wallis Test was used to determine: (i) if the differences in the MIC values of the herbs were significant and (ii) if the differences in the susceptibilities of the M. ulcerans isolates to the various herbal preparations were significant. (2) The isolates from the two BU endemic communities (Amasaman and Nsawam) were grouped separately and the Mann-Whitney procedure was used to determine if the differences in their susceptibilities by community to the herbal preparations were significant. (3) The means procedure, Eta and Eta2, were used to determine: (i) if there was an association between the herbal preparations and their MIC values; (ii) if there was an association between the individual M. ulcerans isolates and their susceptibilities to the herbal preparations and (iii) if there was an association between the BU endemic community isolates and their susceptibilities to the herbal preparations. A P-value of 0.05 was considered significant in the Kruskal-Wallis and Mann-Whitney procedures.

RESULTS

DEMONSTRATION OF ANTI- ACTIVITY

Twenty-five (56.82%) of the 44 herbal preparations inhibited the growth of all the 7 Mycobacterium ulcerans isolates investigated (Table 1). Five (20.00%) of the herbal preparations namely; Tonic 1, Aglaonema commutatum, Zanthoxylum xanthoxyloides , Spathodea campanulata and Gratiola officinalis are purportedly being used for the treatment of Buruli ulcer by the Traditional herbal practitioners (THPs) who provided them.

Figure 1

Table 1a: Test Results Of Herbal Preparations Investigated For Activity Against

Botanical Name/Family & Voucher Number	Ethno medical usage of relevance to study	Plant Part Tested & Effect on *M. Ulcerans* Isolates
1. *Acacia nilotica* MIMOSOIDEAE [GC45912]	Sores	Bark of stem (-)
2. *Ageratum conyzoides* COMPOSITAE [GC45910]	Wound dressing, antimicrobial, skin rashes	Leaves (+)
3. *Aglaonema commutatum* ARACEAE [GC45932]	Buruli ulcer, Antimicrobial	Leaves (+)
4. ***Allium sativum*** (Large/White cloves) LILIACEAE GC [GC45915]	Antimicrobial, anticancer, leprosy, gastric ulcer, TB, antiseptic, scrofulous sores,	Bulb (+)
5. ***Allium sativum*** (Small/Purple cloves) LILIACEAE [GC 45933]	Antimicrobial, anticancer, leprosy, gastric ulcer, TB,	Bulb (+)
6. Aloe vera (syn. *A. barbadensis*) (Thin leaf variety) ALOEACEAE [GC45925]	Antimicrobial, accelerates wound healing, burns, sores, TB, ulcers, psoriasis, acne, anti-inflammatory, seborrhoeic dermatitis, yaws.	Leaves (+)
7. Aloe vera (syn. *A. barbadensis*) (Thick leaf variety) ALOEACEAE [GC45914]	Antimicrobial, accelerates wound healing, burns, sores, TB, ulcers, psoriasis, acne, anti-inflammatory, seborrhoeic dermatitis, yaws.	Leaves (+)
8. *Alstonia boonei* APOCYNACEAE [GC45909]	Antimalarial and antimicrobial	Leaves (+)
9. *Bridelia Ferruginea* EUPHORBIACEAE [GC45896]	Antimicrobial	Bark (+)
10. *Calendula officinalis* ASTERACEAE [GC45934]	Accelerates wound healing, anti-inflammatory, gastritis, eczemas, diaper rash and cradle cap.	Flowers (-)
11. *Capsicum annuum* SOLANACEAE [GC 45902],	Wounds, sores, gastric ulcer, Anti inflammatory	Fruit (+)
12. *Cassia alata* CAESALPINIOIDEAE [GC 45916]	Skin diseases, especially ringworm, antibacterial	Leaves (-)
13. *Cassia occidentalis* CAESALPINIOIDEAE [GC45900]	Anti-inflammatory, antifungal and antimicrobial	Leaves (+)
14. *Cassia ziebertana* CAESALPINIODEAE [GC 45922]	Antimicrobial	Root: (-)
15. *Chalcas exotica* RUTACEAE [GC45927]	Diabetic ulcers, sores, wounds, sickle cell ulcers, inflammation, boils, antimicrobial	Leaves (-)
16. *Chamomilla recutita* COMPOSITAE [Imported]	Insect bites, bruises, frostbite, mouth/gum sores, wounds, antimicrobial, anti-inflammatory	Flowers (-)
17. *Cinnamomum zeylanicum* LAURACEAE [GC45917]	Antimicrobial, antiseptic.	Leaves(-)
18. *Cleome viscosa* CAPPARIDACEAE [GC45898]	Antimicrobial	Leaves (+)
19. *Gratiola officinalis* SCROPHULARIACEAE [GC 45918]	Buruli ulcer, Antimicrobial	Bark (+)
20. *Hydrastis canadensis* RANUNCULACEAE [Imported]	Antimicrobial, skin disease, general ulceration, Anti inflammatory, sore eyes.	Root: (+)
21. *Lannea nigritana* ANACARDIACEAE [GC45894]	Antimicrobial	Root (+)
22. Launaea taraxacifolia COMPOSITAE [GC 45920]	Antimicrobial	leaves (-)
23. *Momordica charantia* CUCURBITACEAE [GC45907]	Antimicrobial,	Leaves(-)
24. Moringa oleifera MORINGACEAE [GC45923]	Antimicrobial	Leaves (-)

Figure 2

Table 1b: Test Results Of Herbal Preparations Investigated For Activity Against

Botanical Name & Voucher Number	Ethno medical usage of relevance to study	Plant Part Tested & Effect on *M. Ulcerans* Isolates
25. *Nauclea latifolia* RUBIACEAE [GC 45921]	Antimicrobial,	Root (-)
26. *Phyllanthus fraternus* EUPHORBIACEAE [GC45901]	Antimicrobial, antiviral cancer, cancerous wounds.	Leaves (+)
27. *Pridium guajava* MYRTACEAE [GC45908]	antimicrobial	Leaves (+)
28. *Pupalia lappacea* AMARANTHACEAE [GC45899]	Chronic sores, boils, skin rashes	Leaves (+)
29. *Sesamum radiatum* [GC45926]	Antimicrobial	Bulb (-)
30. *Solanum torvum* SOLANACEAE [GC45911]	Antimicrobial, haemostatic	Leaves (+)
31. *Spathodea campanulata* BIGNONIACEAE [GC 45935]	Buruli ulcer relief for skin conditions, swollen cheeks, body rashes.	Root (+)
32. *Spigelia anthelmia* LOGANIACEAE [GC45903]	Antimicrobial	Leaves (-)
33. Swedish bitters (cocktail of 11 herbs) [Imported]	Antimicrobial, scars, wounds, cuts, swellings, deformation of the legs, arthritis	Alcoholic extract (+)
34. *Symphytum officinale* BORAGINACEAE [Imported]	Sprains, broken parts, chronic wounds, burns, ulcers, swellings, bruises, cuts, abscesses, gangrene, TB	Root (-)
35. *Syzygium aromaticum* MYRTACEAE [GC 45913].	Antimicrobial, antiseptic, germicide, assists the action of other medicines.	Seed (+)
36. Tonic 1 (cocktail of 2 herbs)	Buruli ulcer, antimicrobial	Leaves & Grains (+)
37. Tonic 2 (cocktail of 2 herbs)	Antimicrobial,	Leaves (+)
38. Tonic 3 (cocktail of 4 herbs)	Antimicrobial, Anticancer	Leaves (+)
39. Tonic 4 (cocktail of 2 herbs)	Antibacterial, antiviral, antimalarial, broadspectrum antimicrobial	Bulbs (-)
40. *Trichilia monadelpha* MELIACEAE [GC45895]	Wound healing, yaws, ulcers, sores.	Bark (-)
41. *Vitellaria paradoxa* SAPOTACEAE [GC45919]	Skin, mucosae, antibiotic, bacteristatic, fungistatic, cutaneous, subcutaneous parasitic infection	Bark (-)
42. *Zanthoxylum xanthoxyloides* RUTACEAE [GC45897]	Buruli ulcer, Broad spectrum antimicrobial agent, anticancer	Roots (+)
43. *Zea mays* GRAMINACEAE [GC45924]	Poultices used for ulcers, rheumatic pains and swellings.	Grains (-)
44. *Zingiber officinale* ZINGIBERACEAE [GC45906]	Antimicrobial, wounds, anti-inflammatory, anticancer,	Bulb (-)

Key: (+): Inhibited the growth of *Mycobacterium ulcerans* isolates
(-): Did not inhibit the growth of *Mycobacterium ulcerans* isolates

Composition of the Tonics

- Tonic 1: *Spigelia anthelmia* (45903), *Zea mays* (45924)
- Tonic 2: *Citrus auratifolia* (45905), *Gossypium barbaense* (45904)
- Tonic 3: *Iathropha curcas* (45931), *Gossypium hirsutum* (45928), *Physalis angulata* (45929), *Delonix regae* (45930)
- Tonic 4: *Sesamum radiatum (45926), Zingiber officinale (GC45906)*

MINIMUM INHIBITORY CONCENTRATIONS OF HERBAL PREPARATIONS

MICs of the 10 herbal preparations are presented in Table 2. Briefly, the MICs were between 0.20 and 12.50% vol/vol (i.e. 1:640 – 1:2 dilution), with Hydrastis canadensis exhibiting on average, the highest inhibitory activity (1.73 ± 1.38% vol/vol), followed by the white variety Allium sativum (2.40 ± 2.0% vol/vol), while Syzygium aromaticum and Spathodea campanulata exhibited the lowest inhibitory activity (12.5 ± .00% vol/vol % vol/vol). The differences in the MICs were statistically significant (P 0.01) and the eta2 coefficient (0.655 = 65.5%) also showed that the herbal preparations greatly accounted for the variations in the MIC values obtained.

Figure 3

Table 2: Minimum Inhibitory Concentrations (% Vol/Vol) Of Herbal Preparations With Antimicrobial Activity Against Isolates

Herbal Preparation	Concentration of herbal preparations expressed as %vol/vol							
	Reference Isolates		Clinical Isolates					Mean MIC & Titre of Herbal Prep.
	Ghana ATCC 970321	Benin ATCC 990826	Amasaman Isolate 1	Amasaman Isolate 2	Amasaman Isolate 3	Nsawam Isolate 1	Nsawam Isolate 2	
1. Tonic 1	12.50	6.25	3.13	3.13	3.13	3.13	3.13	4.91(5.54) [1:2-1:8]
2. Tonic 2	6.25	12.50	12.50	6.25	12.50	6.25	6.25	8.93(5.34) [1:2- 1:4]
3. Tonic 3	12.50	6.25	12.50	3.13	3.13	3.13	3.13	6.25(4.42) [1:2-1:8]
4. *Syzygium aromaticum*	12.50	12.50	12.50	12.50	12.50	12.5	12.50	12.50(.00) [1:2]
5. *Allium sativum* [Large white loves]	1.56	3.13	1.56	3.13	6.25	0.39	0.78	2.40(2.0) [1:4-1:64]
6. *Allium sativum* [Small purple cloves]	3.13	3.13	3.13	1.56	3.13	3.13	3.13	2.91(0.59) [1:8-1:16]
7. *Hydrastis canadensis*	0.78	3.13	3.13	1.56	3.13	0.20	0.20	1.73(1.38) [1:8-1:128]
8. *Zanthoxylum xanthoxyloides*	12.50	12.50	6.25	6.25	6.25	12.50	12.50	9.82(3.34) [1:2-1:4]
9. *Spathodea campanulata*	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.5(.00) [1:2]
10. *Gratiola officinalis*	12.50	12.50	0.78	0.78	1.56	12.50	12.50	7.59(6.13) [1:2-1:32]
Mean Susceptibility of Individual Isolates	8.67(5.14)	8.44(4.43)	6.80(5.10)	5.08(4.31)	6.41(4.45)	6.62(5.32)	6.66(5.27)	
Mean Susceptibility of Isolates by Community			6.10(4.53)			6.64(5.16)		

Key: Mean (SD)
Titre: 1:2= 12.5% vol/vol 1:4= 6.25% vol/vol 1:8= 3.13% vol/vol 1:16= 1.56% vol/vol
1:32= 0.78% vol/vol 1:64=0.39% vol/vol 1:128=0.20% vol/vol 1:256=0.10% vol/vol

SUSCEPTIBILITIES OF ISOLATES TO HERBAL PREPARATIONS

Table 2 shows that all the 7 M. ulcerans isolates were susceptible to each of the herbal preparations, but at different susceptibilities. The average susceptibilities of the 7 M. ulcerans isolates were between 5.08 ± 4.31% vol/vol and 8.67 ± 5.14% vol/vol. The Amasaman #2 isolate was on average the most susceptible (5.085.08 ± 4.31% vol/vol) to the herbal preparations, while the Ghana reference isolate was the least susceptible (8.67 67 ± 5.14% vol/vol). However, the differences in susceptibilities of the 7 isolates were not statistically significant (P 0.05) and were subsequently confirmed by a low eta2 coefficient (0.050 = 5.0%).

SUSCEPTIBILITIES OF ISOLATES TO HERBAL PREPARATIONS BY COMMUNITY

The comparative susceptibilities of the isolates by community presented in Table 2 shows that the Amasaman group of isolates was on average (6.10 ± 4.53% vol/vol) more susceptible to the herbal preparations than the Nsawam group of isolates (6.64 ± 5.17 %vol/vol). However, the difference in their susceptibilities was not statistically significant (P 0.05). The low eta2 coefficient (0.003 = 0.3%) further confirmed the weak effect that the source (community) of the isolates had on their susceptibilities to the herbal preparations.

DEMONSTRATION OF EXTENDED ANTIMICROBIAL ACTIVITY

All the 10 (100%) herbal preparations also demonstrated extended antimicrobial activity against some or all the fast growing microbes investigated, as shown in Table 3.

Figure 4

Table 3: Extended Antimicrobial Profile Of Buruli Ulcer Herbal Preparations

Herbal Preparation	Microbes Inhibited *In-Vitro*
1. *Hydrastis canadensis*	St, Pa, Ca, Sa, Asp, Bsp, Strep, Staph, Bc, Sal, Kp, Ec, Hek [13/19]
2. Allium sativum [White garlic]	St, Ca, Sa, Asp, Bsp, Strep, Sal, Staph, Ec, Hec, Kp, Pa, Bc, Psp, Ef, Sad, Bs, An, Ss [19/19]
3. *Allium sativum* [Purple garlic]	St, Ca, Sa, Asp, Bsp, Strep, Sal, Staph, Ec, Hec, Kp, Pa, Bc, Psp, Ef, Sad, Bs, An, Ss [19/19]
4. *Syzygium aromaticum*	St, Ca, Sa, Asp, Bsp, Strep, Sal, Staph, Ec, Hec, Kp, Pa, Bc, Psp, Ef, Sad, Bs, An, Ss [19/19]
5. Tonic 1	St, Pa, Sa, Asp, Bc, Bsp, Strep, Sal, Staph, Kp Bs, Psp [12/19]
6. Tonic 2	St, Sa, Bc, Bsp, Strep, Sal, Staph, Kp Ef, Ec, Sad, Ss, Bs, Psp [14/19]
7. Tonic 3	Sa, Asp, Bc, Bsp, Strep, Sal, Staph, Psp [8/19]
8. *Zanthoxylum xanthoxyloides*	Staph, Sa, Strep, Bsp, Bc, Psp [6/19]
9. *Spathodea campanulata*	Staph, Sa, Strep, Bc, St [5/19]
10. *Gratiola officinalis*	Bc, Bs, Psp [3/19]
Reference Antibiotic (Pefloxacin mesylate dehydrate)	St, Sa, Bsp, Strep, Sal, Staph, Ec, Hec, Kp, Pa, Bc, Psp, Ef, Sad, Bs, Ss [16/19]
Reference Antifungal (Miconazole)	An, Asp, Ca [3/19]

Key:

Reference isolates
Sa (*Staphylococcus aureus*)
Bc (*Bacillus cereus*)
Ec (*Escherichia coli*)
Psp (*Pseudomonas sp*)
Ef (*Enterococcus faecalis*)
Sad (*Salmonella adabraka*)
Bs (*Bacillus subtilis*)
An (*Aspergillus niger*)

Clinical isolates
Staph (*Staphylococcus spp.*)
Bsp (*Bacillus spp.*)
Hec (**Haemolytic** *E. coli*)
Pa (*Pseudomonas aeruginosa*)
Kp (Klebsiella spp)
St (*Salmonella typhimurium*)
Strep (*Streptococcus spp.*)
Sal (*Salmonella spp*)
Ss (*Shigella sonnei*)
Asp (Aspergillus spp.)
Ca (*Candida albicans*)

DISCUSSION

DEMONSTRATION OF ANTI- ACTIVITY

Given that 56.81% (n=44) of the herbal preparations that were selected on the basis of their purported dermatological and antimicrobial properties also demonstrated anti-M. ulcerans activity, goes to confirm the assertion by several ‘phytoscientists' that the most appropriate and surest way to identify higher plants for drug discovery is by follow-up of their ethnomedical uses (Spieler, 1981; Ogura et al., 1982; Oubre et al., 1997; Fabricant and Farnsworth, 2001). Furthermore, given that 21.74% (n=24) of the effective herbal preparations (Tonic 1, Zanthoxylum xanthoxyloides, Spathodea campunalata, Gratiola officinalis and Aglaonema commutatum) were provided as BU herbal medicines by 3 THPs, lends credence to their claims of successful treatment of BU patients with herbal medicines, while also indirectly confirming reports of successful herbal treatment of BU patients by other THPs. This is a hopeful finding because most of the effective herbal preparations are those commonly used for the treatment of sores, wounds, swellings and ulcers (of other etiological origin) in our traditional settings; suggesting that BU treatment may not be as elusive as we have come to think. The study has primarily shown that there are a number of herbal preparations in Ghana with anti-M. ulcerans activity; hopefully, the same

plants may be found in other African and tropical countries with similar climatic conditions. In view of this plausible assumption, since BU is endemic not only in Ghana but other African and tropical countries (Portaels et al., 1998; WHO, 2001, Van der Werf et al, 2005), the identified herbal preparations should be investigated further since some may be effective in-vivo and consequently help address the large-scale difficulties associated with the treatment of BU. The herbal preparations that we have identified could also offer scientists who are interested in isolating bioactive compounds a wide range of choices for BU drug research and development.

MINIMUM INHIBITORY CONCENTRATION (MIC) OF HERBAL PREPARATIONS

The differences in the MICs of the herbs were statistically significant, suggesting that some of the herbal preparations may be more effective than others; for which reason, there is the need to decide on the herbal preparations that merit further investigation. According to van den Berghe & Vlietinck's (1991) ‘rule of thumb', a prominent plant with antibacterial effect that is worthy of further investigation, is one that apart from the 1:2 dilution, the 1:8 and 1:32 dilutions also show inhibitory activities. Against this background, 3 of the preparations namely; Hydrastis canadensis (1:8 - 1:128), Allium sativum, white variety (1:4 - 1:64) and Gratiola officinalis (1:2 - 1:32) merit further investigation without argument. However, Allium sativum, purple variety (1:8 - 1: 16), Tonic 1 (1:2 – 1:8) and Tonic 3 (1:2 – 1:8) could also be included since they are somewhat within the range. These selections notwithstanding, in-vivo results sometimes fail to support in-vitro findings, therefore there is a risk in adhering strictly to the rule because a selected herbal preparation may fail in-vivo, while a rejected herbal preparation may rather prove effective in-vivo. This is confirmed by the modest performance of Zanthoxylum xanthoxyloides, Spathodea campanulata and Tonic 1 in-vitro, while they are being used effectively in BU patients (personal communication). Anyway, the ultimate guide to treatment of any infection must be clinical outcome; against this backdrop, all the herbal preparations that have demonstrated activity in-vitro should be investigated further in-vivo for identification of ‘the' useful BU herbal preparation. In the interim, the herbal preparations that have been identified in our study could be used as poultices on the lesions, while further studies are concluded regarding the possibility of their systemic application.

SUSCEPTIBILITY OF ISOLATES TO HERBAL PREPARATIONS

All the 6 Ghanaian M. ulcerans isolates (though few) were successfully inhibited by all the 10 herbal preparations, suggesting that other Ghanaian M. ulcerans isolates may also be susceptible to the same batch of herbal preparations. Secondly, the differences in susceptibilities amongst the individual isolates on one hand and between the communities on another hand were negligible; a finding that was also confirmed by the eta squared coefficients which showed that the successful inhibition of the M. ulcerans isolates was largely due to the herbal preparations (0.655 = 65.5%) and negligibly due to the individual isolates (0.050 = 5.0%) and the communities (0.003 = 0.3%), 0.3%) from which the isolates were obtained. These findings suggest that herbal therapy should not be overlooked in the search for an effective BU treatment since it may hold some promise. An effective herbal medicine would provide a window of hope for both the accessible and affordable treatment of Buruli ulcer because herbal preparations are usually cheaper than orthodox drugs and can be prepared by the users themselves in most cases. This approach could enhance efforts at controlling the disease since those mostly affected by BU reside in areas where health facilities are far away and therefore not easily accessible. Besides, most BU patients occupy the lower rungs of the socio-economic ladder and as such have difficulty accessing the health services available, especially surgery ($710/case) (Amofa et al, 2002), which is currently the main treatment option (Thangaraj et al., 1999). Herbal therapy could be considered, provided the herbal preparations are found to be safe in pre-clinical studies. Preclinical safety studies may suffice for some of the herbal preparations since there are reports that herbal therapies are already being used; moreover, there is also an urgency to find treatment for BU since its prevalence is higher than that of tuberculosis and leprosy in some communities (Amofa et al., 2002). In the case of herbal preparations without supportive history, clinical trials would have to be conducted after the herbal preparations have been found to be effective and safe in pre-clinical studies.

Herbal therapy would be worth considering as a treatment option because at some stage of BU (in both BU patients and experimentally infected animals), a large number of bacilli are present (Portaels et al., 1998; Addo et al., 2005, 2007), suggesting that a favourable condition for the selection of resistant mutants could be created. It is for this reason that combination therapy (as practised in the treatment of TB) has been adopted in the treatment of BU (WHO, 2003;

WHO, 2004; Etuaful et al., 2005). Some TB drugs have also been found to be effective to some extent in BU treatment and have therefore been recommended by W.H.O as the current antibiotic treatment for BU (WHO, 2003). Unfortunately the use of the TB drugs (ex. rifampicin and streptomycin) could lead to an increase in drug resistance, especially in undiagnosed TB patients, which could eventually result in an increase in TB cases, especially in TB/BU endemic communities. In view of this, herbal therapy should be actively pursued as a BU treatment option because a single herb is never a single compound but a group of compounds, which potentiate each other or create synergy (Stermitz et al., 2000). In other words, the use of a single herb could simulate combination therapy, which may prevent, or at worse, delay the development of antimicrobial resistance. Against this background, the herbal preparations that we have identified should be used as whole plants to preserve their 'combination therapy' property, since a lot of plant extracts are known to fail because only the active ingredient is isolated, which leads to the loss of the synergy that is conferred by the rest of the plant components (Chaudhury, 1992; Duke et al., 1998; Stermitz et al., 2000). Several cases have been documented by Beckstrom-Sternberg & Duke (1994), where synergy was lost by using the single-ingredient approach to develop drugs from plants.

DEMONSTRATION OF EXTENDED ANTIMICROBIAL ACTIVITY

Observations in BU patients and M. ulcerans infected animals demonstrate that M. ulcerans disseminates into regional lymph nodes, visceral organs and bone marrows, with resultant osteomyelitis, (Portaels et al., 1998;, Addo et al., 2005; Addo et al, 2007), which is often secondarily infected by other microorganisms (WHO, 2001; Addo et al, 2007); while some BU conditions also become gangrenous (WHO, 2001; Addo et al., 2005) and cancerous with time (WHO, 2001). Therefore, the supplementary demonstration of broadspectrum antimicrobial activity by all the 10 herbal preparations investigated (especially against organisms with predilection for the skin such as Staphylococcus aureus, Staphylococcus spp., Pseudomonas aeruginosa, Bacillus cereus, Bacillus spp and Candida spp, Aspergillus spp) makes them desirable BU remedies. The use of a single herbal preparation with activity against both M. ulcerans and other microbes will simultaneously treat the M. ulcerans infection and some of the accompanying complications if caused by the microbes that they successfully inhibited. Above all, the identified herbal preparations could be combined if found to be synergistic, in order to obtain better and lasting performance.

In conclusion, this study has shown that there are a number of herbal preparations in Ghana with anti-M. ulcerans activity in-vitro, and a few others (provided by THPs who are using them for treating BU patients) with anti-M. ulcerans activity in humans. These findings both directly and indirectly respectively, confirm reports that herbal medicines are being used to treat BU in Ghana. Since BU is endemic in Ghana and other African countries (Portaels et al., 1998; WHO, 2001), and it is also currently without an effective and affordable treatment, the identified herbal preparations should be investigated with all seriousness since some may help address the difficulties associated with the treatment of BU; especially since they also posses extended antimicrobial activity which may simultaneously treat secondary infections that are often associated with BU. We appreciate that the isolates and the communities studied were very few, and as such a larger number of isolates and communities have to be investigated to generalize. That notwithstanding, the 10 herbal preparations have shown effectiveness at inhibiting the growth of M. ulcerans in-vitro and it has been demonstrated statistically that the successful inhibitions were largely determined by the herbal preparations and very minimally influenced by the isolates or sources of the isolates, therefore suggesting that the herbal preparations investigated may be effective against other Ghanaian M. ulcerans isolates. Above all, herbal therapy should be investigated further since the BU herbal preparations provided by traditional herbal practitioners successfully inhibited all the M.ulcerans isolates investigated, suggesting that herbal therapy holds some promise and could eventually be considered as one of the BU treatment options, especially in the face of the possible development of resistance to TB drugs; one of the current treatment options. Lastly, in view of the occasional absence of correlation between in-vitro and in-vivo findings, pre-clinical (safety/efficacy in-vivo) and clinical studies may have to be undertaken to select a suitable herbal preparation from among those identified in-vitro in our study.

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