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Management of Diabetic Bacterial Foot Infections with Organic Extracts of Liverwort *Marchantia debilis* from Cameroon

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Diabetic bacterial foot infections (DBFIs) are limb-threatening complications in patients with diabetes mellitus, accounting for 50% of diabetes related lower limb amputations in developing countries, representing approximately 20 percent of all diabetes-related hospital admissions with significant healthcare-related costs involved. The widespread problem of bacterial resistance to most commonly used antibiotics places a huge economic burden on the healthcare system, with both increased morbidity and mortality among diabetic patients with foot infections. In this study, the antibacterial activity of organic extracts of the fresh liverwort *Marchantia debilis* from the North West Region of Cameroon is reported. An exit pool system, where patients presenting with DBFIs consented to be involved in the use of phytomedicines, after long term treatment of ulcers with antibiotics and not yielding significant long term benefit, presented themselves at the Phytobiotechnology Research clinic (PRF). Continuous culture of swabs from foot and toe wounds from 30 infected patients on nutrient agar and MacConkey agars in triplicate as well as Gram stain microscopy, revealed the presence of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis* and *Bacillus* species. Light petroleum and methanol extracts of the whole bryophyte plants at 100% concentration were tested. *In vitro* inhibition of the tested bacterial isolates from the diabetic foot ulcers by *M. debilis* was observed only with the light petroleum extract. No inhibition by the extracts was observed for the *Pseudomonas aeruginosa* isolate. The light petroleum extract of *M. debilis* was formulated into a petroleum oil based cream named BryoCream™. This was administered to 20 of the patients with 90% cure rate in a three week time period. The main nonpolar components were determined by GCMS as lepidozene and β-barbatene, and by NMR as stigmasterol and β-sitosterol. In conclusion, nonpolar extracts from bryophytes from Cameroon could, potentially, be used to treat diabetic bacterial foot infections.

Keywords: *Marchantia debilis*, Liverwort, Diabetic, Foot, Bacteria, Infection.

Many bioactive compounds have been isolated from bryophytes, especially liverworts originating from Asia, Europe and South Africa [1-3]. For example, Adio *et al.* [1] and Allison and Chud [3] identified a number of such compounds from liverworts in New Zealand. Volatile constituents have been identified in liverworts including *Tritomaria polita*, *Marsupella emarginata*, *M. aquatica* and *M. alpina*.

Asakawa and colleagues [4-6] reported that bryophytes are potentially rich in compounds such as sesqui- and diterpenoids, bibenzyls and bisbibenzyls, as well as flavonoids, with allergenic contact dermatitis, antimicrobial, antifungal, cytotoxic, insect repellent, insecticidal, molluscidal, cardiotonic and muscle relaxing activities. Bryophytes are very common across Africa, particularly in Cameroon.

Diabetes mellitus (DM) is projected to affect a staggering figure of 552 million people in 2030 as compared with 371 million in 2011. A few decades ago, DM was considered as a disease of affluent societies. Today, it is known that most of the world's diabetes patients live in developing countries [7-9]. As such, with the recent rising incidence of this condition, the developing world, the African continent, including Cameroon, has been hard hit by this pandemic, and is experiencing an exponential rise in its incidence, as well as its burdens. Again, estimates suggest a giant leap from 14 million diabetics in 2012 to 28 million for sub-Saharan Africa in 2030, with as many as 85% of the cases largely undiagnosed. The WHO African Region describes the impact and devastating outcomes of diabetes in the region as one of Africa's New Silent Killers.

Treatment of diabetic foot infections in sub-Saharan Africa and throughout the world is very challenging due to slow healing and increasing antibiotic resistance [10-12]. The drugs of choice for the systemic treatment of DFIs are mostly cloxacillin, ciprofloxacillin, and azithromycin and gentamicin [13-15], and topically using bacitracin powder and antiseptics such as alcohols [16-18]. These drugs of choice and antiseptics are proving to be ineffective for sustained DFI treatment. Antibiotic resistance in clinical treatment of diseases in Africa is rising exponentially [19-22]. Consequently, the need to explore alternative antimicrobials for DFIs is exigent [23-28]. Plants, particularly lower plants, which have often been ignored, could provide new drug leads for the treatment of DFIs. The ecology of Cameroon is rich in algae, hornworts, lichens, mosses and liverworts, especially since the country lies in the Congo forest and with Mount Cameroon harboring a rich biodiversity of both lower and higher plants. A quick survey of

bryophytes in Cameroon reveals many unidentified species, with familiar species such as *Marchantia* sp. The main objective of this study was to provide a preliminary report on the application of the light petroleum extract of the liverwort *M. debilis* from Cameroon in the treatment of DFIs, both *in vitro* and *in vivo.*

Table 1 shows the various bacterial isolates that are prevalent among the patients presenting with DFI. *Staphylococcus aureus* was the most frequently isolated in this study, followed by *Pseudomonas aeruginosa*. Most of the isolates that make up DFBI among the patients were Gram-negative bacterial infections. These bacteria are generally resilient and easily tolerate antibiotics. The isolated bacteria in this study and their distribution are very important, as many general medical practitioners make prognostic treatments of DFIs without proper culture of the exudates and proper etiologic agents, as well as a rigorous antibiotic sensitivity profile. The infective organisms of DFIs in Africa have not been well studied, and the microbial isolates may not be known thoroughly.

Table 1: DBFI isolates from 30 patients with bacterial frequency in percent.

DBF isolate from patients	Frequency of occurrence on 30 patients (%)
Staphylococcus aureus	45
Proteus mirabilis	10
Bacillus sp.	5
Pseudomonas aeruginosa	25
Escherichia coli	15

In earlier investigations, several sesquiterpenes and sterols have been detected in extracts of *Marchantia* species [4]. Asakawa [4-7] reported the isolation and structural determination of secondary metabolites of several hundred species of bryophytes (including *Marchantia* sp.) collected from all over the world and a number of interesting biological activities were reported of sesqui- and diterpenoids and complex aromatic compounds like bisbibenzyls, as mentioned earlier.

Table 2: Antibacterial activity of the petroleum and methanol extracts of *Marchantia debilis* as zones of inhibition (Average of duplicates in mm).

Extract	*S. aureus*	*E. coli*	*P. aeruginosa*	*Bacillus* sp.
Light petroleum extract	15	12	0	17
Light petroleum, control	15	12	0	17
Methanol extract	0	0	0	0
Methanol control	0	0	0	0

In this work our goal was to test one nonpolar (light petroleum) and one polar (methanol) extract of *M. debilis*. The results show that the light petroleum extract possessed antibacterial ingredients (Table 2), whereas the methanol extract was inactive. This suggests that the active ingredients are nonpolar compounds. GCMS analyses revealed two dominant components in the nonpolar (diethyl ether) extract of *M. debilis*: lepidozene and β-barbatene. Analyzing the ^{13}C NMR spectra of different fractions of the *n*-hexane extract of *M. debilis,* beside fatty acids, showed stigmasterol as the most dominant component of the sterol fractions; β-sitosterol was also recognized [33]. Although it is known that stigmasterol and β-sitosterol possess antibacterial and anti-inflammatory activity [34-37] we cannot ascribe to them the effectiveness of bryocream since they were present in the methanol extract as well, which was not active. Further investigation is necessary.

The secondary metabolites of bryophytes from Africa, including Cameroon, have not been widely studied. In other studies using higher plants and mushrooms, Yongabi *et al.* [29, 30] reported the activity of a number of medicinal plant extracts on yeast isolates from clinical specimens from HIV patients in Cameroon. Table 3 shows that the bryophyte based preparation was effective (90%) in the treatment of DFIs among patients in Cameroon.

Table 3: *In vivo* evaluation of Bryocream and ointment on patients with DFIs at PRF clinics.

Kind of DFIs	No. of patients	No. cured	Percentage cured* (%)	Duration of treatment (days)	Status
Foot ulcers	20	18	90	7-10	All diabetic

*With a patient whose wound got completely dried.

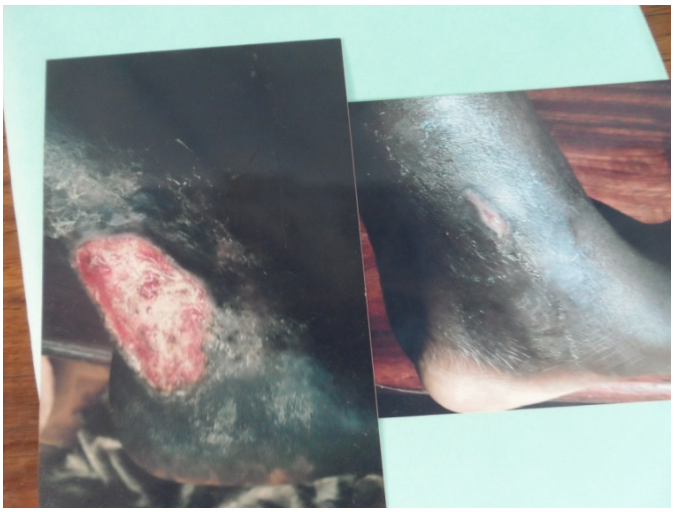

Figure 1: DFI ulcer (left), and after treatment with Bryocream and ointment (right) after 14 days.

Figure 1 shows a classical foot ulcer that was treated in this study in about 14 days when both the cream and ointment was applied daily. This is promising, and is the first report on the use of the bryophyte flora of Cameroon to attend to diabetic bacterial foot infections. The conclusion is drawn that these bryophyte medicated products may offer a comparatively cheaper, safer and effective approach to managing diabetic foot bacterial infections in sub-Saharan Africa and more detailed screening for full exploitation is recommended.

Experimental

Criteria for specimen collection: The diabetic patients opted to be treated using naturopathic therapy that was developed at the PRF research station. PRF is a registered research non-Governmental Organization with the Cameroon Government and is registered with the European Union. Specimen collection and tests were based on the complaints given by the patients, as well as para-clinical examinations. Patients without symptoms and signs of diabetes, and without a DFI were excluded from this study. Ethical consideration for this study was given by the CBC Health Board in Cameroon, with approval number IRB 2013-10, August 3, 2013.

Specimen collection: The patients were all diabetic, whose status was confirmed at the Bamenda regional hospital. All were receiving glucophage, insulin and metformin, and were re-confirmed at the PRF Clinics using the onestop glucose testing kit, a commercial kit. Thirty diabetic patients through an exit pool were considered. Swabs were collected from the affected wounds and ulcers using sterile swab sticks and the specimens appropriately labeled using a bold marker and serial number assigned to represent each patient's name.

Microscopic examination and culture of swabs and necrotic debris: The specimens were appropriately processed using the Gram stain method [9], and portions of each specimen were cultured aseptically by streaking onto nutrient and MacConkey agars and incubated at room temperature at 37ºC for 36 h according to established methods [9, 29]. Plates were examined for their morphology and micro morphology [30].

Sources, identification and processing of collected bryophytes: *M. debilis* Goebel, was collected from the banks of streams around raffia bushes at w3Mendakwe, Bamenda, North West region of Cameroon. Voucher specimen was taken and identified by biologists at CCast Bambili and confirmed by Y.A. The plant material was carefully washed, followed by crushing in a mortar using a pestle, and passed through sieves of 3 mm mesh. Voucher samples were stored at the Phytobiotechnology Research Laboratory, Bamenda, Cameroon.

Extraction procedures

Extraction for making biocream: Liverwort (50 g) was added separately to methanol and light petroleum, 250 mL (1:5, w/v) in beakers, and allowed to extract for 72 h at room temperature [13, 29]. The extracts were filtered through Whatman filter paper no 1 (Whatman, UK) and the filtrate was evaporated under vacuum at 38ºC. The resulting dried extracts were stored in sterile, screw capped bottles and kept at room temperature.

Extraction for GCMS analysis: Dried plant material was carefully purified, crushed in a mortar using a pestle, and extracted with diethyl ether for 30 min. An ultrasonic bath was used for the first 5 min of extraction. After extraction, the extract was filtered through filter paper and then through a glass pipette filled with silica gel to remove traces of water. After dilution with *n*-hexane, the solution was analyzed by GCMS conducted on an Agilent Technologies 6890N gas chromatograph coupled with a mass detector (Agilent Technologies 5973), provided with a DB 5 (30 m × 0.25 mm ID × 0.25 μm d_f) capillary column. The analyses were performed in EI mode (70eV) using He at 1 mL/min. The injection temperature was set at 250ºC. The analyses were carried out using a temperature program starting from 50ºC with an initial 5 min hold to 250ºC, with a 10ºC/min heating increase and keeping the final temperature stable for 20 min. The mass range was set at *m/z* 40-500 with 3 scans. The transfer line was set at 280ºC. Co-injection of the extracts with C9-C25 hydrocarbons was performed under the same conditions.

Extraction for NMR analysis: Dried and purified plant material was crushed in a blender and extracted with *n*-hexane for 24 h. An ultrasonic bath was used for the first 1 h of extraction. After extraction, the extract was filtered through a filter paper to remove the remaining plant material. NMR spectra were recorded on a Varian 500-PS NMR instrument at 500 MHz for ^{1}H and 125 MHz for ^{13}C in $CDCl_3$.

Determination of antibacterial activity of the extracts: The agar diffusion method according to Yongabi and colleagues [32] was employed. A sample (0.2 g) of the *M. debilis* extract was reconstituted in distilled water (5 mL). Antibiotic susceptibility was determined by the agar well diffusion method as commonly used and standardized in the US by the National Committee for Clinical Laboratory Standards (NCCLS). The zone of inhibition was measured and the results interpreted as sensitive, intermediate resistant, or resistant. The zone sizes of inhibition were measured and interpreted using the NCCLS, as recommended by WHO [9]. Each extract was incorporated in a 6 mm well previously bored using a steel borer. Controls were set up by introducing the extraction solvents (methanol and light petroleum) into different wells. The plates were incubated at 37ºC for 36 h. The development of inhibition by the extracts against the test organism was measured [9, 29, 30, 31] The differences between the inhibition rates of the extracts, the test, and that of the control were recorded as the actual diameter of zones of inhibition caused by the extract [31, 32]. The solvents, methanol and light petroleum did not exhibit any inhibition in this study.

Preparation of bryophyte-based cream: The organic extracts (200 mg) of *M. debilis* were blended into 200 g of Petroleum jelly following standard chemical techniques reported previously by Yongabi [31].

Preparation of bryophyte extracts based ointment using olive oil base: The organic extracts (200 mg) of *M. debilis* were blended into olive oil and palm kernel oil (200 mL). Standard organic chemistry protocols, as described by Yongabi *et al.* [30] were applied.

Administration of bryophyte cream and ointment to patients recruited in the study versus placebo group: Twenty diabetic patients from PRF clinics and other local clinics in the North West Region of Cameroon were enrolled for management of their wound with Bryocream™, a *M. debilis* extract based therapy, developed at the Phytobiotechnology Research Clinic, Bamenda, Cameroon. Patients' wounds were cleaned through swabbing with distilled water, and with no antiseptics, as traditionally done. Appropriate culture tests for proper etiologic identification were made before the products were applied. A positive control cream formulated with methanol and light petroleum was also used. The 20 patients were properly educated on the mode of administration and asked to report on a weekly basis for observation and follow up. Each patient received a screw capped bottle containing a cream and another containing the ointment. A sample (20 g) of the cream was administered daily after application of the ointment (5 mL) for a 3 weeks maximum therapeutic period. Follow up was terminated when the infection wound dried up. It was resolved that the patients should present for re-examination at our clinic periodically.

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