Antibacterial activities of Millipede extracts against selected bacterial pathogens

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Received 24 May 2015
Accepted 1 July 2015

ABSTRACT

Objective: In the search for potent antimicrobial agents against bacteria and other microbial infections this study evaluated the antibacterial properties of millipedes extracts against selected control strains of bacterial pathogens including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*.

Methods: Two species of millipedes were identified as *Ophistreptus guineensis* S. (Diplopoda: Spirostreptidae) and *Pachybolus ligulatus* V. (Diplopoda: Pachybolidae) were collected from the University of Ghana Botanical Gardens and placed into 3 groups - Group 1 = *O. guineensis* alone, Group 2 = *P. ligulatus* alone, and Group 3 = mixture of *O. guineensis* + *P. ligulatus*. The millipedes were killed, dried, and grounded into coarse powders. Chloroform, ethanol, aqueous extracts were prepared and screened for activity using agar well and paper-disc diffusion assays. Minimum inhibitory and bactericidal concentrations (MIC and MBC) of the most active extracts were also evaluated by the broth micro-dilution technique.

Results: Ethanol extracts of Group 3 (mixture of *O. guineensis* + *P. ligulatus*) showed the highest activity, with mean diameters of zones of inhibition of 29 ± 0.02 and 14 ± 0.00 mm against *S. aureus* and *E. coli* respectively. No activity was observed for Group 2 extracts (*P. ligulatus* alone), while some level of activities were observed for Group 1 extracts (*O. guineensis* alone). MIC and MBC values of 4.9 and 25.0 mg/ml of the ethanol extract of Group 3 were found against *S. aureus*.

Conclusions: Work is in progress to isolate the bioactive agent(s) in millipede extracts.

KEY WORDS: **Antibacterial**
Extract
Millipedes
MIC
MBC

Introduction

Millipedes belong to the phylum Arthropoda, subphylum Myriapoda, and class Diplopoda. The most prominent characteristic of millipedes are the presence of a head, and externally segmented body containing two pairs of legs on each segment. The name millipede is a compound word derived from the Latin root '*mille'* (thousand) and '*pes'* (foot). Common species of millipedes have elongated cylindrical bodies, and between 36-400 legs. Most species of millipedes feed on decaying plant materials (detritivorous), while a few species are omnivorous, and carnivorous [1].

Millipedes are one of the several ingredients used in traditional medicine in certain parts of the world. In the Yoruba culture of Nigeria, crushed millipedes are used to treat fever, whitlow, and convulsion in children [2]. The juice and smashed millipede pulp have also been reported to be used to treat wounds and earaches in Cameroun and Zambia [3]. Many millipede species emit various poisonous liquid secretions which include alkaloids, benzoquinones, phenols, terpenoids, and hydrogen cyanide [4]. In Mexico, dry diplopods are made into powder and applied as a plaster around affected parts in order to treat joint illnesses [5].

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Antibacterial resistance continues to grow quickly among key bacterial pathogens all over the world [6-7]. Development of new antibacterial agents is therefore imperative. Hence, there is the need for potent antibacterial agent(s) against pathogenic bacteria. Plants and animals are valuable sources of natural products and in most parts

of the world, are used as chemotherapeutic agents. For example, extracts from centipedes and millipedes are used to treat many conditions in China and other parts of the world [8]. Nevertheless extracts of millipedes in Africa have not been adequately studied for their antimicrobial activities. Therefore this study was designed to evaluate antibacterial properties of millipede extracts against selected standard control strains of bacterial pathogens.

Materials and methods

Collection and processing of millipedes

Ethical clearance (Number: SAHS-ET./10346606/AA/38A/2013-2014) was obtained from the Ethics and Protocol Review Committee, School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences, University of Ghana before the start of the study. Live millipedes were collected from the University of Ghana Botanical Gardens, Legon, Accra. The collected millipedes were placed in sterile containers, and transported to the Department of Animal Biology and Conservation Science (DABCS), School of Biological Sciences, University of Ghana, and taxonomically identified as the African Pumpkin or Ghana Chocolate millipede, *Ophistreptus guineensis* S. (Diplopoda: Spirostreptidae) and the African Banded Amber millipede, *Pachybolus ligulatus* V. (Diplopoda: Pachybolidae) by the first author (MKB). The millipedes were later transported to the Microbiology Laboratory (ML), SBAHS, Korle-Bu, Accra for further analyses. At the ML, samples were placed into 3 groups - Group 1 = *O. guineensis* alone, Group 2 = *P. ligulatus* alone, and Group 3 = mixture of *O. guineensis* + *P. ligulatus*. The millipedes were killed, cut into pieces, dried under sunlight for 3-4 days and crushed into powders using a grinding stone. The powders were weighed and placed into air-tight containers to avoid the absorption of moisture. Voucher specimens of the collected millipedes were placed at the DABCS Museum, School of Biological Sciences, University of Ghana, for future reference.

Extraction from the powdered millipedes

Extracts of the millipedes were prepared using modifications of the methods by Pesewu *et al*. [9-10]. Each of the dry grounded millipede powders (50 g) were placed in 500 ml beakers, 200 ml of chloroform, ethanol, and distilled water (H_2O) were added to each beaker to form separate mixtures of the solvent extracts. The mixtures were left to soak for 48 h, with intermittent shaking. After the 48 h period, the mixtures were agitated vigorously for 10 min, allowed to settle for 5 min and the supernatant liquid were passed through Whatman No. 1 filter paper (Whatman International Limited, UK) to remove solid millipede materials. For the chloroform and ethanol extracts, the solvents were evaporated from the filtrates in *vacuo* at 37°C in a Buchi Rotavapor rotary evaporator (Rose Scientific Limited, Canada) whiles the aqueous extracts were freeze dried using a Modulyo freeze dryer (Thermo Fisher Scientific, USA). Dry weight of each extract was expressed as a percentage of the dry weight of the original millipede powder used. To prepare stock solutions of the millipede extracts, the chloroform and ethanol extracts were first dissolved in dimethyl-sulphoxide (DMSO), while aqueous extracts were dissolved in distilled H_2O and sterile filtered through 0.2 µm Millipore filters (Merck Millipore Corporation, Germany) prior to usage.

Tested bacteria and media used

Reference control strains of bacteria used in the study were obtained from the American Type Collection Culture (ATCC), USA and the National Collection of Type Cultures (NCTC), UK. The control bacteria included: *Staphylococcus aureus* (NCTC 6571), *Pseudomonas aeruginosa* (ATCC 19429), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumoniae* (ATCC 13883). The test bacteria were maintained on nutrient agar slants prior to use. Media used for the antibacterial evaluation of the millipedes extracts were: Nutrient agar (NA), Iso-Sensitest broth (ISB), and Mueller-Hinton agar (MHA) obtained from Oxoid Ltd, Basingstoke, Hampshire, UK. Media were prepared according to the manufacturers specifications.

Screening for antibacterial activity

Antibacterial activities of the millipede extracts were compared with standard reference control antibiotics, using the agar-well and paper-disc diffusion assays. Tests were based on guidelines of the Clinical Laboratory Standard Institute (CLSI) [11] and British Society for Antimicrobial Chemotherapy (BSAC) standards [12].

Pure cultures of the test bacteria were inoculated onto MHA plates. For the agar well assay, wells (6 mm in diameter) were punched into inoculated MHA agar plates and each of chloroform, ethanol or aqueous millipede extracts (100 µl) were added into the different wells.

Standard paper discs of gentamicin (10 µg) obtained from Axiom Laboratories, India were used as antibiotic controls. In the paper-disc diffusion assay, paper discs were prepared with the various solvent extracts of the millipedes according to the method by Cheesbrough [13], and placed onto the surface of inoculated MHA plates with the aid of sterile inoculated pins. All inoculated plates with wells and paper discs prepared were left on the bench for 15 min to allow diffusion of the extracts and antibiotic controls before incubating for 24 h at 37°C. After the overnight incubation, the diameter of the inhibition zones around the wells and control discs were measured with vernier calipers. All tests were repeated 3 more times and the mean diameters of inhibition zones calculated.

Quantitative antibacterial activity evaluation

Minimum inhibitory concentration (MIC) of the ethanol extract of Group 3 (mixture of *O. guineensis* + *P. ligulatus*) of the millipedes against the reference control strains of bacteria used in the study was determined according to the methods of CLSI [11] in 96-well microtitre trays. Concentrations of the ethanol Group 3 millipede extract were prepared by serial doubling dilutions and tested against the bacteria in a range of 100-0.098 mg/ml. To determine the minimum bactericidal concentration (MBC), a set of NA plates were prepared and 20 µl of the cultures from each of the wells, the controls were sub-cultured onto the NA plates and then incubated at 37°C for 48 h. The lowest concentration that did not show growth of the test bacteria on sub-culture was taken as the MBC of the millipede extract.

Statistical analysis

Data obtained from the experiments were entered into a database and analyzed statistically using analysis of variance (ANOVA) and descriptive statistics such as means and percentages. p values < 0.05 were considered as statistically significant differences.

Results

Extracts

The % yields of Group 1 (*O. guineensis* alone) ranged from 1.60-3.30, while Group 2 (*P. ligulatus* alone) ranged from 2.20-5.10 (Table 1). With Group 3, (mixture of *O. guineensis* + *P. ligulatus*) the % yields ranged from 2.00-4.72. The lowest % yields were observed in the aqueous extracts of all the millipedes ranging from 1.60-2.20. However, the highest % yield was found for the ethanol extract of Group 2 (5%), giving an indication that ethanol may be more effective in the extraction of millipede powders than the other solvents.

Table 1. Dry weights (g) and percentage (%) yields of the various groups of millipede extracts

Groups	Solvents	Dry weights	Percentage yield(s)
1	Chloroform	1.20	2.40
1	Ethanol	1.65	3.30
1	Aqueous	0.80	1.60
2	Chloroform	1.43	2.86
2	Ethanol	2.55	5.10
2	Aqueous	1.10	2.20
3	Chloroform	1.05	2.10
3	Ethanol	2.36	4.72
3	Aqueous	1.00	2.00

Group 1= *O. guineensis* extracts alone; Group 2= *P. ligulatus* extracts alone; Group 3= mixture of *O. guineensis* + *P. ligulatus* extracts

Antibacterial evaluation

Mean diameters of zones of inhibition of the millipede extracts against selected bacterial pathogens are presented in Tables 2 and 3. From the tables, the highest inhibition activity of the millipede extracts (29 ± 0.02 mm) was observed in the ethanol extract of Group 3 (mixture of *O. guineensis* + *P. ligulatus*) against *S. aureus* in the agar well diffusion assay. The lowest activities were observed for the aqueous extracts of the millipedes in both the agar-well and paper-disc diffusion assays (Tables 2 and 3). None of the extracts of Group 2 (*P. ligulatus* alone) were active against any of the test bacteria by both agar-well and paper-disc diffusion assays. However, all the extracts of Group 1 (*O. guineensis* alone) showed some level of activity against the test bacteria including *S. aureus* and *E. coli* (Tables 2 and 3). For the agar-well diffusion assay, mean diameters of zones of inhibition ranging from 11-25 ± 0.02 mm for the extracts of Group 1 (*O. guineensis* alone) against the test bacteria were found. Also extracts of Group 3 (mixture of *O. guineensis* + *P. ligulatus*) showed mean diameters of zones of inhibition ranging from 10-29 ± 0.01 mm against the test bacteria used in the

study. Ethanol extract of the Group 3 (mixture of *O. guineensis* + *P. ligulatus*) showed slight activity against *E. coli*, with mean diameters of zones of inhibition ranging from 10-14 ± 0.02 mm. It must be noted that none of the millipede extracts investigated were active against *Ps. aeruginosa* and *K. pneumoniae* in both the agar well and paper-disc diffusion assays (Tables 2 and 3). Mean diameters of zones of inhibition ranging from 13-29 ± 0.00 mm by the control antibiotics were observed against the test bacteria (Table 3).

Table 2. Mean diameter of zones of inhibition (mm) for the various extracts of the millipedes and the commercially prepared control antibiotic using agar-well diffusion assay.

Group	Solvent	Test bacteria			
		S. aureus	***Pa***	***E. coli***	***Kp***
1	Chloroform	18 ± 0.01	N/A	N/A	N/A
1	Ethanol	25 ± 0.00	N/A	10 ± 0.00	N/A
1	Aqueous	11 ± 00	N/A	N/A	N/A
2	Chloroform	N/A	N/A	N/A	N/A
2	Ethanol	N/A	N/A	N/A	N/A
2	Aqueous	N/A	N/A	N/A	N/A
3	Chloroform	20 ± 0.01	N/A	N/A	N/A
3	Ethanol	29 ± 0.02	N/A	14 ± 00	N/A
3	Aqueous	10 ± 0.05	N/A	N/A	N/A
Gentamicin (10 µg)		22 ± 0.30	26 ± 0.20	29 ± 0.20	24 ± 00

Pa = Pseudomonas aeruginosa; *Kp =Klebsiella pneumoniae*; N/A = Not Applicable

Quantitative antibacterial evaluation

MIC and MBC values of 4.9 mg/ml and 25 mg/ml were obtained in the ethanol extract of Group 3 (mixture of *O. guineensis* + *P. ligulatus*) against *S. aureus* in the study (Table 4). MIC and MBC values of 0.0092 mg/ml and 1.0934 mg/ml of the standard control antibiotic (ciprofloxacin) were obtained against *S. aureus*. Of all the test bacteria used in the study, MIC and MBC values of the millipede extract were recorded for *S. aureus* only (Table 4).

Table 3. Mean diameters of zones of inhibition (mm) for the various extracts of the millipedes and the commercially prepared control antibiotics using the paper-disc diffusion assay

Group	Solvent	Test bacteria			
		S. aureus	***Pa***	***E. coli***	***Kp***
1	Chloroform	10 ± 0.01	N/A	N/A	N/A
1	Ethanol	12 ± 0.00	N/A	10 ± 0.00	N/A
1	Aqueous	8 ± 10	N/A	N/A	N/A
2	Chloroform	N/A	N/A	N/A	N/A
2	Ethanol	N/A	N/A	N/A	N/A
2	Aqueous	N/A	N/A	N/A	N/A
3	Chloroform	N/A	N/A	N/A	N/A
3	Ethanol	15 ± 0.02	N/A	10 ± 0.01	N/A
3	Aqueous	N/A	N/A	N/A	N/A
Controls					
Cotrimoxazole (5 µg)		13 ± 0.03	24 ± 0.12	26 ± 0.04	23 ± 0.30
Tobramycin (15 µg)		N/A	28 ± 0.15	29 ± 0.18	25 ± 0.16
Gentamicin (10 µg)		22 ± 0.30	26 ± 0.20	29 ± 0.20	24 ± 00
Tetracycline (30 µg)		21 ± 0.20	N/A	N/A	22 ± 01

Pa =*Pseudomonas aeruginosa*; Kp =*Klebsiella pneumoniae*; N/A = Not Applicable

Table 4. MIC and MBC values (mg/ml) of ethanol extract of millipede (Group 3[a]) against the bacterial isolates.

Test bacteria	Group 3 extract		Control*	
	MIC	**MBC**	**MIC**	**MBC**
S. aureus	4.9	25.0	0.0092	1.0934
Ps. aeruginosa	NA	NA	NA	NA
E. coli	NA	NA	NA	NA
K. pneumoniae	NA	NA	NA	NA

[a,] mixture of *O. guineensis* and *P. ligulatus* extracts
*Ciprofloxacin

Discussion

Nowadays due to the alarming rate of antimicrobial resistance among bacteria and other microorganisms, there have been calls for the development of new antimicrobial agents. It has been reported that millipedes produce chemicals such as phenolic compounds, alkaloids, quinones, terpenoids, and organic acids which may show antibacterial activity [14]. In view of this, the extracts of the millipedes were investigated for antibacterial activity against the test bacteria. In the present study, *S. aureus* was more susceptible to the millipede extract, thus producing the highest mean diameter of zone of inhibition (27 ± 0.15 mm) by the agar-well diffusion assay (Table 2). The activity observed with the extract may be due to certain com-

ponents found in the tissues and bodies of the millipedes. Previous reports indicate that 2 compounds from the segmental secretions of the benzoquinone-producing Yellow-banded or bumble bee millipede, *Anadenobolus monilicornis* von P. (Diplopoda: Rhinocricidae) and *Orthoporus dorsovittatus* V. (Diplopoda: Spirostreptidae) have both been found to be used by the Wedge-capped capuchin monkeys or Weeper capuchin, *Cebus olivaceus* S. (Primates: Cebidae) in self-anointing themselves in order to deter mosquitoes and other insects from biting the monkeys [15-18]. Antifungal activities of the defensive secretions of certain millipedes of the family Xystodesmidae, including *Cherokia georgiana georgiana* B., *Cleptoria rileyi* B., *Euryurus maculatus* K., and *Oxidus gracilis* K. have been reported in the U.S.A. by Roncadori et al. [19]. Anti-sickling and antibacterial activities of extracts from *Tachypodoiulus* sp. (Diplopod: Julidae) have also been reported in Congo [20]. MIC values of 0.062 mg/ml and 0.125 mg/ml of organic acids and alkaloids of millipede extracts against *S. aureus* have been previously found [20]. Therefore it can be proposed that *S. aureus* strains were more sensitive to the millipede extracts than *E. coli*, *Ps. aeruginosa*, and *K. pneumoniae*. Moreover, the MIC and MBC values for the control antibiotic, which was ciprofloxacin, was very low compared to the ethanol millipede extract used in the present investigation (Table 4).

It must however be noted that the millipede extract was a crude one and that if it is purified; the antibacterial activity might be greater or less than the control commercial antibiotics used in the study. From the study, extracts of *O. guineensis* and a mixture of *O. guineensis* + *P. ligulatus* have shown some levels of antibacterial activity against the selected bacterial pathogens *in-vitro*. Work is in progress to isolate, purify, identify the bioactive agent(s) in the millipede extracts, using various analytical methods including the high performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) techniques.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgements

We thank the staff of the Department of Medical Laboratory Sciences (MEDLAB), School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences, and the Department of Animal Biology and Conservation Science (DABCS), School of Biological Sciences, University of Ghana for the roles they played during the study.

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