EVALUATION OF ANTIMICROBIAL PROPERTIES OF THE RUSTY MILLIPEDE *TRIGONIULUS CORALLINUS*

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ABSTRACT

Over the years, the use of lower organisms and their products have played massive role as effective resources in pharmacotherapy. In the present study, the antimicrobrial activity of various solvent extracts of the whole body of the rusty millipede *Trigoniulus corallinus* was evaluated. The whole body was extracted using non polar solvents (hexane, petroleum ether and chloroform), polar protic solvents (butanol, ethanol, methanol, water and saline) and polar aprotic solvents (acetone and ethyl acetate). Antimicrobial activities were carried out by standard disc diffusion method. The highest mean diameter of zone of inhibition (18±0.5 mm) was observed against bacterial pathogen *Pseudomonas aeruginosa* and highest mean diameter of zone of inhibition (21±0.07 mm) was observed against the fungal pathogen *A. flavus* in the acetone extract. Among the ten different solvent extracts tested, antimicrobial activity was clearly prominent in the ethyl acetate extract followed by acetone extract. While, the ethyl acetate extracts of the rusty millipede could inhibit the growth of all the tested pathogenic bacteria and fungi, acetone extracts could inhibit the growth of all the tested microbes except bacterium *Enterococcus faecalis*. When the aqueous extracts of the millipede *Trigoniulus corallinus* could inhibit the growth of none of the tested microbes, the ethanol extract could inhibit the growth of just one pathogenic fungi and the petroleum ether extract could inhibit the growth of just one pathogenic bacteria. Together, the results of this study demonstrate, for the first time, the antimicrobiological effects of the rusty millipede, *Trigoniulus corallinus* which seems to possess therapeutic molecules capable of inhibiting, the growth of all the pathogenic bacteria and fungi.

Key Words: *Trigoniulus corallinus*, pathogens, whole body extracts, antimicrobial activity

INTRODUCTION

The resident animals of polluted areas possess possibly novel antimicrobials to be free from infections. In arthropods especially myriapods have two immune strategies: (a) the cellular immune response by haemocytes and other tissues resulting in phagocytosis of smaller foreign

bodies and nodule or capsule formation around larger xenografts and (b) the humoral defence, which occurs extracellularly but is normally cell-mediated [1]. Antimicrobial peptides (AMPs) are also known as major components of innate immune defense system in invertebrates [2]. In addition, invertebrates have strategies to defend themselves against foreign organisms, by production of secondary metabolites that repel them. The first line of defense of arthropods against pathogens and parasites is of physical nature *via* their hard cuticle. However, once this barrier is passed, a complex interaction of innate humoral and cellular immune reactions is induced in both tissues and haemocoel, which results in a fast elimination of micro-organisms [3]. Millipedes (Myriapoda, Diplopoda) produce defensive fluids which are secreted through multiple ozopores, arranged laterally along the body. The fluids are largely believed to act as a deterrent to predators, but may also act as antimicrobials protecting the hosts from many pathogens within the detritus in which they feed (including carrion). Black lemurs use these millipede defensive fluids as potential antiparasitics, biting the millipede to induce toxin secretion then rubbing these toxins into their fur [4]. Many millipede species emit various poisonous liquid secretions which include alkaloids, benzoquinones, phenols, terpenoids and hydrogen cyanide [5]. Benzoquinones are a class of organic molecule that may have use as novel anticancer agents [4]. In Mexico, dry diplopods are made into powder and applied as a plaster around affected parts in order to treat joint illnesses [6]. Antimicrobial activity was against gram-positive and gram-negative bacteria have also been described [7]. The present study was undertaken to assess the antimicrobial property of the rusty millipede *Trigoniulus corallinus* against pathogenic bacteria and fungi.

MATERIAL AND METHODS

Millipede collection: Live specimens of the millipede *Trigoniulus corallinus* was collected from the swampy areas and coconut groves of Holy Cross College (Autonomous), Nagercoil, Kanyakumari District, TamilNadu, India.

Preparation of tissue extracts: The whole body of the millipede was weighed and extracted with different solvents such as ethanol, methanol, butanol, chloroform, ethyl acetate, acetone, hexane, petroleum ether, saline and distilled water. For extraction, solvents were added at the ratio of 10 g/20 ml and were shaken at regular time intervals for three days at room temperature for thorough extraction of the biologically active compounds from the whole body. The contents were filtered using Whatman No. 1 filter paper and the filtrate was concentrated by allowing the solvent to

evaporate at room temperature. The concentrated extract was used as solvent extract of the sample and stored in refrigerator (-20ºC).

Microbial strains used: Antimicrobial activity of whole body extract was determined against six human pathogenic bacteria viz., *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis* and three fungal strains viz., *Aspergillus niger*, *A. flavus* and *Rhizopus stolonifer*.

Assay of antibacterial activity: Antibacterial activity of the whole body extracts in the different solvents of the millipedes *Trigoniulus corallinus* were tested using standard disc diffusion method of Bauer [8]. Sterilized Muller Hinton Agar (20 ml) was poured into sterile petriplates and allowed to solidify. After solidification, 100 µl of fresh culture of pathogenic bacteria were spread on the surface of Muller Hinton Agar plates. Sterile disc of 6 mm, loaded with 25 µl of whole body extract as well as positive (streptomycin disc, 25µg/disc) and negative control (sterile disc) discs for comparison were placed in the plates. The plates were incubated for 24 h at 37ºC. After incubation the diameter of zones of inhibiton formed around each discs were measured (mm) and recorded.

Assay of antifungal activity: Sterilized potato dextrose agar (20 µl) was poured into sterile petriplates and allowed to solidify. After solidification, 100 µl of fresh culture of pathogenic fungal strain was distributed uniformly on the surface of potato dextrose agar plate with the help of sterile cotton swab. The sterile disc of 6 mm, loaded with 25 µl of crude extract as well as Flucanozole (100 µg / disc) used as positive control and sterile disc used as negative control were placed in the fungal plates and were incubated at 27°C for 48 h and the antifungal activity was measured based on the diameter of the zone of inhibition formed around each disc.

RESULTS AND DISCUSSION

Recently, the invertebrates attract the attention of researchers due to the presence of pharmacologically active drugs and some of these active drugs have been identified and characterized [9]. Kuwahara *et al*., [5] reported that millipedes produce chemical substance such as phenolic compounds, alkaloids, quinones, terpenoids and organic acids which may show antimicrobial activity. In view of this, the extracts of the millipedes were investigated for antimicrobial activity against the test pathogens.

Antibacterial activity of whole body extracts

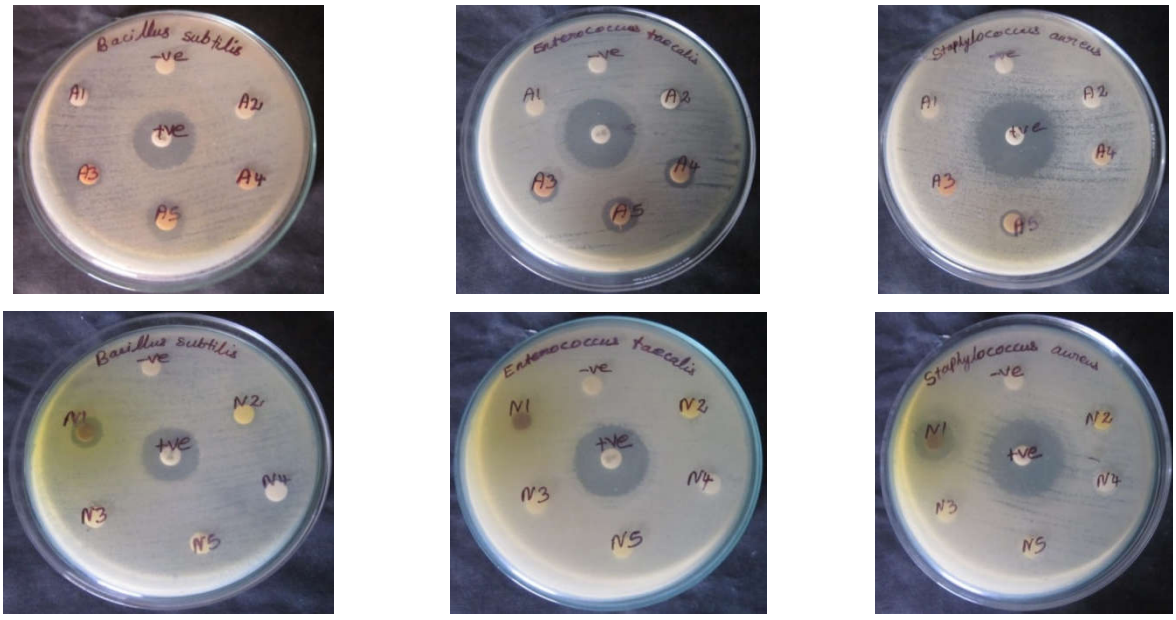
The whole body extract of the millipede *Trigoniulus corallinus* showed different antibacterial activity on different bacteria tested. Among the six different bacterial strains tested, the ethyl acetate extract of the whole body inhibited the growth of all the microbes. The acetone extract showed highest inhibition against *Pseudomonas aeruginosa* (18±0.5 mm), ethyl acetate extract against *Bacillus subtilis* (15±0.5 mm), chloroform extract against *Enterococcus faecalis* (12±0.2 mm), butanol extract against *Enterococcus faecalis* (11±0.05 mm) (Table 1 and Plate 1). The antibacterial activity was previously reported in other millipedes *Pachybolus ligulatus* [10], *Anadenobolus monilicornis*, *Orthoporus dorsovittatus* and *Tachypodoiulus* sp [11, 12], *Pachyiulus hungaricus* [13]. The bacterial inhibition may be due to the synergic action of the antibacterial substances on peptidoglycan of bacterial cell wall to reduce the structural strength leading to degradation, inactivity and death of the bacteria. Bacterial encounter may accelerate immune response in the millipede resulting in the production of antimicrobial substance causing the destruction of the pathogen. Probably, the antibacterial substances synthesized from the hemocytes of the millipedes [1] or from the defensive gland, the ozadenes [14] and secreted out through the ozopores protect the host millipede.

Table 1 Antibacterial activity of the whole body extracts of the millipede *Trigoniulus corallinus* against pathogenic bacteria

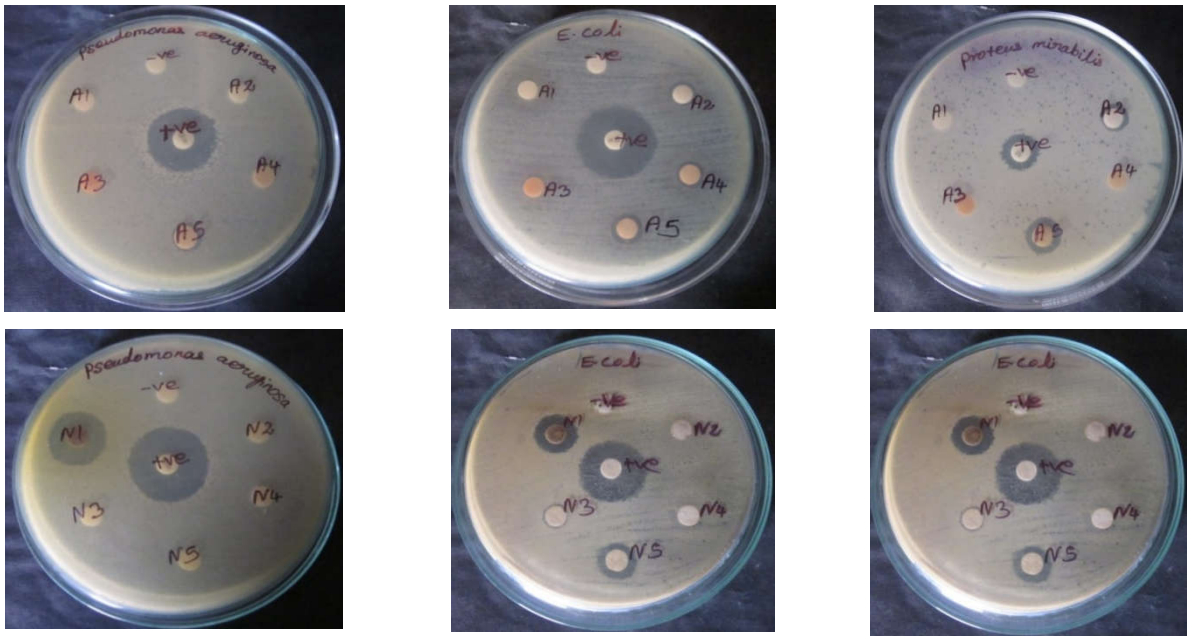
Extracts	Zone of inhibition (mm)					
	Bacillus subtilis	***Enterococcus faecalis***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Proteus mirabilis***
Ethanol	-	-	-	-	-	-
Methanol	-	7±0.09	-	-	-	8±0.7
Butanol	9±0.1	11±0.05	-	-	-	-
Aqueous	-	-	-	-	-	-
Saline	-	-	-	-	12±0.9	-
Ethyl acetate	15±0.02	13±0.05	9±1.01	8±0.09	12±0.05	12±0.03
Acetone	11±0.5	-	13±0.4	**18**±0.5	10±0.5	11±0.02
Chloroform	-	12±0.2	-	-	9±0.01	-
Hexane	-	-	-	-	-	9±0.05
Petroleum ether	-	-	-	-	7±0.5	-
Streptomycin	18±0.1	25±0.5	28±0.01	23±0.02	25±0.05	13±0.01
Sterile disc	-	-	-	-	-	-

Plate 1 Antibacterial activity of the millipede *Trigoniulus corallinus*

Bacillus subtilis ***Enterococcus faecalis*** ***Staphylococcus aureus***



Pseudomonas aeruginosa ***Escherichia coli*** ***Proteus mirabilis***



Note: A1-Ethanol, A2-Methanol, A3-Butanol, A4-Chloroform, A5-Ethyl acetate, N1-Acetone, N2-Hexane, N3-Petroleum ether, N4-Aqueous, N5-Saline, +ve-Streptomycin, -ve-Sterile disc

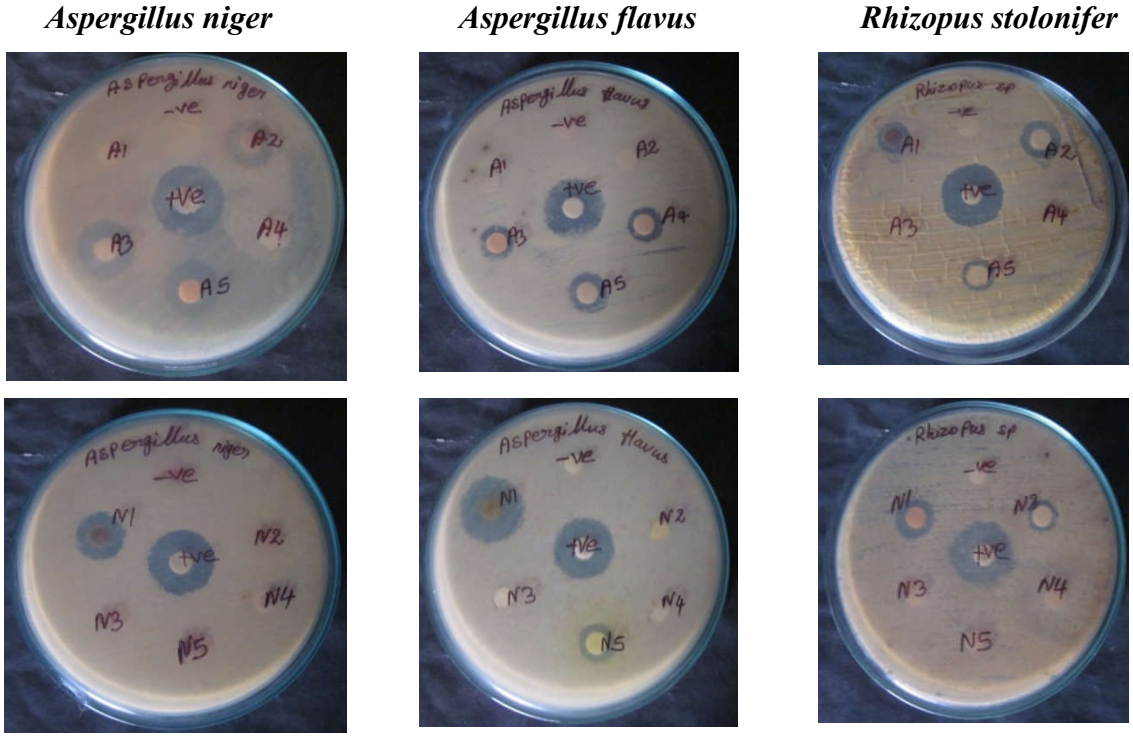
Antifungal activity of whole extracts

Of the three fungal pathogens tested the acetone and ethyl acetate extract of the whole body of the rusty millipede *Trigoniulus corallinus* inhibited the growth of all the three fungi. The highest inhibitory zone was observed in the acetone extract against *Aspergillus flavus* with a inhibitory zone of 21±0.07 mm (Table 2; Plate 2). The antimicrobial protein/peptide found in the extract may alter the configuration of chitin or eliminate/damage the polymer of the chitin the polysaccharide found on the cell wall of the fungus or it may be an agglutinin like carbohydrate binding protein that attach to the chitin of the fungal cell wall to inhibit the fungal activity. Roncadori *et al*., [15] reported the defensive secretions of certain millipedes *Cherokia georgiana georgiana*, *Cleptoria rileyi*, *Euryurus maculatus* and *Oxidus gracilus* with high antifungal property by suppressing mycelial growth and spore germination. Resistance against the fungus *A. flavus* was reported in *Pachyiulus hungaricus* [13]. Organic acids and alkaloid in the crude extracts of *Tachypodoiulus* sp has promising antibacterial activities [16]. Among the samples, the extracts extracted using petroleum ether, water and hexane failed to inhibit the growth of all the three fungi. Fungi are more resistant than the bacterial strains to the tested compound [17]. This could be attributed to the nature of fungal cell wall chitin which is relatively resistant.

Table 2 Antifungal activity of the whole body extracts of the millipede *Trigoniulus corallinus* against pathogenic fungi

Extracts	Zone of Inhibition (mm)		
	Aspergillus niger	***Aspergillus flavus***	***Rhizopus stolonifer***
Ethanol	-	-	12±0.06
Methanol	15±0.02	-	15±0.53
Butanol	17±0.05	11±0.02	-
Aqueous	-	-	-
Saline	-	12	-
Ethyl acetate	16±0.05	14±0.3	11±0.2
Acetone	10±0.9	**21**±0.07	14±0.1
Chloroform	-	12±0.05	-
Hexane	-	-	-
Petroleum ether	-	-	-
Streptomycin	21±0.1	20±0.09	21±0.21
Sterile disc	-	-	-

Plate 2 Antifungal activity of the millipede *Trigoniulus corallinus*



Note: A1-Ethanol, A2-Methanol, A3-Butanol, A4-Chloroform, A5-Ethyl acetate, N1-Acetone, N2-Hexane, N3-Petroleum ether, N4-Aqueous, N5-Saline, +ve-Streptomycin, -ve-Sterile disc

CONCLUSION

These results of the present investigation enforce the idea that diplopods are a source to be considered in the discovery of new substances for drug development to control microbial diseases. Of the various solvents used for the process of extraction, the extracts prepared using ethyl acetate and acetone the dipolar aprotic solvents uncover maximum inhibitory activity against all the tested pathogenic microbes. Further investigations intending to purify these bioactive compounds should be considered to clarify their chemical nature.

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