

Evaluation of *Bacillus cereus* and *Bacillus pumilus* metabolites for anthelmintic activity

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ABSTRACT

Objective: To assess the anthelmintic activity of *Bacillus cereus* and *Bacillus pumilus* metabolites. **Materials and Methods:** The successive solvent extractions with petroleum ether, ethyl acetate and methanol. The solvent extracts were tested for anthelmintic activity against *Pheretima posthuma* at 20 mg/ml concentration. The time of paralysis and time of death of the worms was determined for all the extracts. Albendazole was taken as a standard reference and sterile water as a control. **Results:** All the sample extracts showed significant anthelmintic activity in paralyzing the worms comparable with that of the standard drug. The time of death exhibited by BP metabolites was close to the time exhibited by standard. **Conclusion:** The study indicates both bacteria *Bacillus cereus* and *Bacillus pumilus* have anthelmintic activity indicating potential metabolites in them.

Key words: Anthelmintic, *Bacillus cereus*, *Bacillus pumilus*, *Pheretima posthuma*

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INTRODUCTION

Helminthes infections are among the most common infections in humans, affecting a large population of the world. About 1/3rd of world's population harbors helminthes of one species, or another.^[1] The intestinal roundworms, including hookworms and whipworms, harm the host by depriving him of food, causing blood loss, injury to organs, intestinal or lymphatic obstruction and by secreting the toxins.^[2,3] These infections have led to lowering of immune systems for HIV, malaria and tuberculosis and debilitating the individual both physically and cognitively.^[4] Majority of infections are generally limited to tropical regions and contribute to the prevalence of malnutrition, anemia, eosinophilia and pneumonia.^[5]

Internal parasitic infection is a great threat not only to humans but also to the productivity of the sheep and goat industry.^[6] It has a great impact on the economy of domesticated livestock throughout the world due to their adverse effect on productivity.^[7]

Tremendous progress has been made in the development of anthelmintic drugs in the past 50 years. During

this period, the current classes of synthetic drugs were developed including the benzimidazoles and imidazothiazoles. Another major step was achieved with the introduction of the avermectin class of macrolactones in the early 1980's. The discovery of this compound class led to anthelmintics drugs, such as ivermectin and doramectin, which have excellent broad spectrum activity and superior potency.^[6]

Though many drugs are currently available in the market, still nematodes represent a serious threat to both plants and animals. Resistance to all of these classes of drugs has been observed. New methods of control of these parasites are being sought since a number of soil applied commercial nematocides are being phased out and resistance to anthelmintics is an increasing problem.^[8] The resistance to nematodes has been reported from several countries.^[9,10] Hookworms and some other parasitic nematodes have shown signs of resistance to albendazole, the current treatment approved by the World Health Organization.^[4] The increasing prevalence of helminthes parasites that are resistant to convectional anthelmintics has been the spur for different research programs exploring alternative approaches to control the parasites and to discover new classes of anthelmintics, especially those with a novel mode of action.^[6,11,12]

For much of our past history forages, plant parts or extracts have been used to combat parasitism and in many

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part of the world such natural products are still in use for this purpose. Many potential phytotherapeutics have been revealed that some plant species, when grazed or fed as conserved material may reduce the degree of internal parasite infection in sheep.^[13-15]

Though, the development of the avermectin class of compounds obtained from a soil bacterium *Streptomyces avermitilis* initiated more research to look for microbial metabolites for potent anthelmintic compounds, less literature are available regarding microbial metabolites as anthelmintics. Hence in the present study, the metabolic extracts of two bacteria *Bacillus cereus* (BC) and *Bacillus pumilus* (BP) were tested for anthelmintic activity *in vitro*.

MATERIALS AND METHODS

Solvent extraction and preparation of samples

The two bacteria BC and BP were grown separately in large quantity in nutrient broth medium and incubated for 3 days at 35°C. The broth was centrifuged to separate the cells at 10,000 rpm for 20 min. The clear supernatant containing the metabolites was collected. The metabolites of both organisms were subjected to successive solvent extraction with petroleum ether, ethyl acetate and methanol (1:1) in a separating funnel. All the three solvent extracts were dried in separate plates. The BC petroleum ether extract was labeled as sample BC-1, ethyl acetate extract as BC-2 and methanol extract as BC-3. Similarly the BP extracts were labeled as BP-1, BP-2 and BP-3 respectively.

The adult Indian earthworms were used for the initial screening of anthelmintic compounds *in vitro*.^[16,17] The assay was performed on adult earthworm *Pheretima posthuma*, due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings and also because of its easy availability.^[18-20] The worms were collected from moist soil and washed with normal saline to remove all dirt and fecal matter. The earthworm of 8-12 cm in length and 0.3-0.5 cm in width were selected for study. The worms were divided into eight groups of six worms each. The worms of groups 1-3 were released into separate plates containing a solution of 50 ml of the sample extracts of BC, BC-1, BC-2 and BC-3 respectively. Similarly, the worms of groups 4-6 were released into the sample extracts of BP, BP-1, BP-2 and BP-3 respectively. All the samples were prepared in sterile water at a dose of 20 mg/ml. The group 7 worms were released into 50 ml of standard drug albendazole solution of 20 mg/ml concentration. The control group was placed in plain sterile water of

50 ml (group 8). The assay was carried as per the method of Ajaiyeoba *et al.*^[21]

Observations were made for the time taken to paralysis and death of individual worms. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously and failed to revive even after transfer to normal saline. Death was concluded when the worms lost their motility completely and failed to respond even after a touch with the needle followed by fading away of their body colors.

RESULTS

Bacillus cereus

The sample extracts BC-1, BC-2 and BC-3 paralyzed the worms in 15 min, 17 min and 15 min respectively at 20 mg/ml concentration. The time exhibited by sample extracts for paralysis was better when compared to the standard drug albendazole, which took 30 min for paralysis at 20 mg/ml.

The time taken for death of worms by sample extracts BC-1, BC-2 and BC-3 was 120 min, 130 min and 150 min, respectively. The time taken for death by all the sample extracts was more when compared with standard drug which brought the death in 70 min.

Bacillus pumilus

The sample extracts BP-1, BP-2 and BP-3 paralyzed the worms in 13 min, 16 min and 10 min respectively at 20 mg/ml concentration. This time response by sample extracts for paralysis was much better than the standard drug Albendazole, which took 30 min for paralysis at 20 mg/ml.

The time taken for death of worms by the sample extracts BP-1, BP-2 and BP-3 was 90 min, 85 min and 95 min, respectively. The time taken for death by all the sample extracts was more when compared to standard drug, which killed the worms in 70 min.

The time taken for paralysis of worms and subsequent death in the presence of different sample extracts, and standard drug are shown in Table 1. The histogram showing the paralysis of worms in minutes is shown in Figure 1. The histogram showing the death of worms in minutes is shown in Figure 2.

DISCUSSION

The aim of studies reported here was to test the potential of BC and BP metabolites for anthelmintic activity.

The motive behind to carryout anthelmintic activity was the toxicity shown by the extracts during cytotoxicity studies on various cell lines. The toxicity on cancer cell lines of certain sample extracts was more when compared to toxicity on normal cell lines.

Many bacteria and fungi have been reported to have antinematode activity. Among bacteria the metabolites of genera *Pasteuria*, *Pseudomonas*, *Serratia*, and among fungi *Arthrobotrys*, *Catenaria* and *Cephalosporium* are reported to have antinematode activity.^[22] “Lachnum B1 and B2”, “mycorrhizin B1 and B2”, both brominated metabolites obtained from ascomycete *Lachnum papyraceum* with nematocidal and antimicrobial activities have been reported by Stadler et al.^[23] “3-hydroxypropionic acid” obtained from endophytic fungi *Phomopsis phaseoli* and *Melanconium betulinum* with nematocidal activity against *Caenorhabditis elegans* and *Meloidogyne incognita* has been reported by Schwarz et al.^[24]

“Avermectins”, a series of highly potent anthelmintic and insecticidal compounds of the macrolide structural

class produced by the soil bacterium “*S. avermitilis*” was reported by Burg et al.^[25] Avermectin A, a macrolide, with a molecular weight of 873 doltons is a commercially available compound with anthelmintic and insecticides activity. Since the discovery of the avermectins, members of the structurally and functionally similar milbemycin family have been isolated from *Streptomyces hygroscopicus* subsp. *aureolacrinusus*^[26] *Streptomyces cyaneogriseus* subsp. *moncyogenus*^[27] and *Streptomyces* sp. Strain E225.^[28]

A solvent-extractable metabolite with a small molecular weight, <10,000 Dolton, produced by a novel strain of BP with pesticidal activity against rootworm, beet armyworm and nematodes have been reported by Heins et al.^[29] A crystal protein isolated from soil bacterium *Bacillus thuringiensis* has been reported to kill the nematode worms and it is said to be safe for vertebrates.^[30]

The present investigations have shown that both the bacterial extracts have significant anthelmintic activity. The paralyzing capacities of the sample extracts are comparatively better than the standard drug albendazole. Among the entire sample extracts the methanol extract of BP was good enough to paralyze the worms in 10 min when compared to standard drug time of 30 min. However, regarding the time taken for death of worms, the sample extracts of BC took more time when compared to sample extracts of BP. The time taken by BP extracts for death of worms (between 85 and 95 min) was close to that of the standard drug time of 70 min.

Since the sample extracts of BC showed early paralysis, but delayed death time of the worms, the effect of the extracts may be due to chloride ion conductance of worm muscle membrane, producing hyperpolarization and reduced excitability that leads to muscle relaxation and flaccid paralysis. The BP sample extracts showed not only early

Table 1: Anthelmintics activity of BC and BP metabolites

Sample extracts	Concentration (mg/ml)	Time taken for paralysis (in min)	Time taken for death (in min)
Control (sterile water)	-	240±0.16	300±0.35
Standard (albendazole)	20	30±0.12	70±0.28
BC-1	20	15±0.16	120±0.83
BC-2	20	17±0.15	130±0.93
BC-3	20	15±0.18	150±0.15
BP-1	20	13±0.28	90±0.66
BP-2	20	16±0.05	85±0.88
BP-3	20	10±0.25	95±0.43

Readings are in value (n=6) and SEM. BC: *Bacillus cereus*, BP: *Bacillus pumilus*, SEM: Standard error of the mean

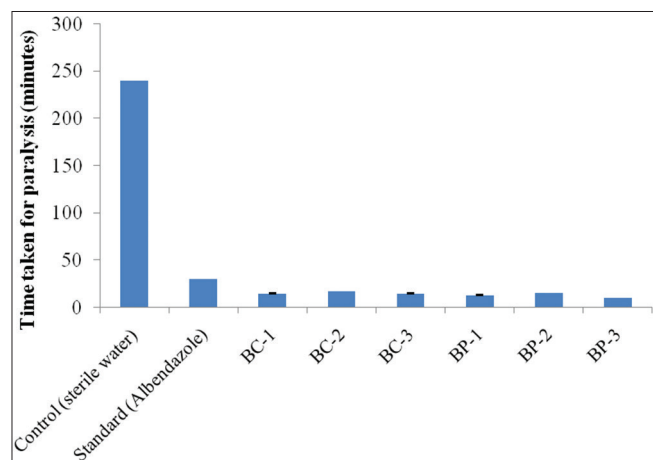


Figure 1: Paralysis of worms in minutes in presence of *Bacillus cereus* and *Bacillus pumilus* metabolites in comparison with standard. BC: *Bacillus cereus*; BC-1 Petroleum ether extract; BC-2 Ethyl acetate extract; BC-3 Methanol extract. BP: *Bacillus pumilus*; BP-1 Petroleum ether extract; BP-2 Ethyl acetate extract; BP-3 Methanol extract

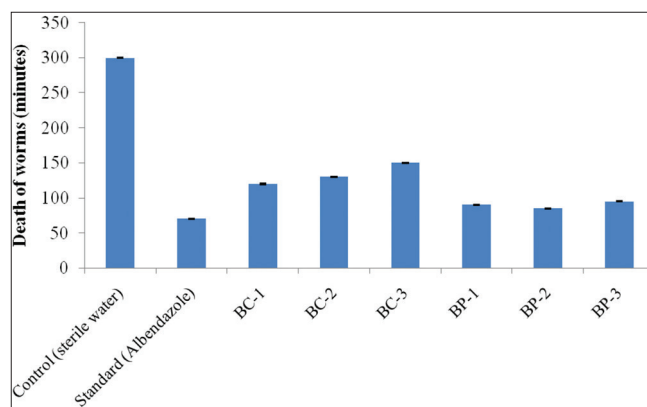


Figure 2: Death of worms in minutes in presence of *Bacillus cereus* and *Bacillus pumilus* metabolites in comparison with standard. BC: *Bacillus cereus*; BC-1 Petroleum ether extract; BC-2 Ethyl acetate extract; BC-3 Methanol extract. BP: *Bacillus pumilus*; BP-1 Petroleum ether extract; BP-2 Ethyl acetate extract; BP-3 Methanol extract

paralysis, but also the early death of worms, which was close to the time of death exhibited by standard drug. Hence, the metabolic compounds of BP responsible for anthelmintic activity appears to be different and also with a different mechanism of action from that of BC metabolites.

Though the BP strain (15.1), with antinematodal activity has been reported by Heins *et al.*^[29] The BC strain with anthelmintic activity has not been reported in the past. Hence, as per the available literature and to the best of our knowledge, this may be the first report on BC bacteria with anthelmintic activity.

CONCLUSION

The present study concludes that the petroleum ether extract, ethyl acetate extract and methanol extract of both bacteria BC and BP have exhibited anthelmintic activity indicating potential metabolites in them. The future scope involves the need of isolation of active constituents responsible for the activity. Further studies are required to establish a mechanism of action.

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