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Anthelmintic and Antibacterial Activity of Red Pigment from *Aspergillus terreus.*

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ABSTRACT

The intracellular red pigment isolated from filamentous fungi, *Aspergillus terreus* grown on starch casein nitrate agar (SCN) was investigated to determine its anthelmintic and antibacterial activity. The extracted crude pigment was characterized using TLC, HPTLC and UV Visible spectroscopy. The egg hatch assay was found to be effective at a concentration of 1.5625 mg/ml of the pigment. The cessation of larval motility of the *Haemonchus contortus* was best observed within 60 min at 50 mg/ml of the pigment concentration and was found to possess even with the sample concentration of 1.5625 mg/ml with a little more prolonged time interval of 150 min. Better antibacterial activity was exhibited against gram negative organisms, *Salmonella typhimurium* and *Escherichia coli* with zones of inhibition of about 15 mm each and *Klebsiella pneumoniae* with an inhibition zone of 10 mm at concentration of 100 µg/ml of the crude pigment sample. The MIC was also evaluated using micro dilution assay by tetrazolium salts. Hence further investigations to isolate the lead molecules in the crude pigment may promise a natural anthelmintic agent which can be used in this era of synthetic anthelmintics to minimize the risk of anthelmintic resistance and it could also be substantiated that the pigment possesses significant antibacterial property.

Keywords: Antibacterial, Anthelmintic, *Aspergillus terreus*, *Haemonchus contortus,* MIC.

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INTRODUCTION

There is a growing interest in microbial pigments due to their natural characteristics, safety, and medicinal properties. Many fungal pigments were isolated in different environments exhibiting interesting medical applications. Natural compounds are a source of numerous therapeutic agents. Recent progress to discover drugs from natural sources has resulted in compounds that are being developed to treat cancer, resistant bacteria and viruses and immunosuppressive disorders. *Aspergillus terreus* is a cosmopolitan fungus which produces pale yellow to brown with yellow soluble pigments that are frequently present. The famous statins, drug lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), for lowering cholesterol to prevent cardiovascular disease is mainly produced by *A*. *terreus* [1, 2]. A series of compounds such as terreineol [3], terreulactone A [4], terrain [5], terreic acid [6] and aspulvinones [7] were also isolated from this fungus.

Helminthosis affect the health and welfare of livestock and compromises the efficacy of production. The grazing animals pick up infection from the pasture and maintain the infection live among the fellow livestock. The highly persistent problem of anthelmintic resistance, the cost of the presently used anthelmintics and the lesser number of newer drugs developed against helminthosis form major concern in the livestock industry [8]. Gastrointestinal helminthosis is controlled mainly by synthetic chemical anthelmintics, which has the disadvantages of being costly, risk of environmental pollution and development of resistant populations. Anthelmintics derived from natural sources like plants and microbes can be a solution to this world wide problem as they form safe and non-toxic agents with an altered site of action [9, 10]. Haemonchus contortus, a gastrointestinal nematode usually found in small ruminants, causes large economic losses to livestock breeders by causing appetite depression, damages in gastric function and alterations in total protein content, energy and mineral metabolism [11].

Natural pigments possess anticancer, antimicrobial, antioxidant and many other biological activities, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH. It has been reported earlier that some pigments produced by microorganisms have the ability to prevent the growth of other bacteria. A few investigations have already been conducted to compare the antibiotic activities of organisms isolated from different origins [12, 13, 14].

In the present study we focus to discuss on the anthelmintic activity using *Haemonchus contortus* ova and larvae and antibacterial activity against pathogens of human and animal importance of the red colored pigment from *Aspergillus terreus*.

MATERIALS AND METHOD

Collection of soil sample

The soil sample was collected from sediments of Banasura sagar dam located at Wayanad district, Kerala which is the second biggest dam in Asia made up of mud.

Isolation and characterization of *Aspergillus terreus*

The soil sample collected was air dried and the organism was isolated by serial dilution technique using starch casein nitrate medium (SCN) (g/l) (agar- 15, soluble starch- 10, Potassium phosphate dibasic- 2, Potassium nitrate- 2, Sodium chloride- 2, Casein- 0.3, $MgSO_4$. $7H_2O$- 0.05, $CaCO_3$- 0.02, $FeSO_4$. $7H_20$- 0.01g) (pH-7.2) supplemented with chloramphenicol using spread plate method. The plates were then incubated at 30 °C for two weeks.

The isolated red pigmented fungal culture was characterized macroscopically and microscopically using slide culture method [15]. It was subject to molecular identification by partial sequencing of 5.8s rRNA at IMTECH, Chandigarh and was subject to BLAST search analysis. The culture was also screened for its pigment production in various fungal growth medium like Potato Dextrose Agar (PDA), pH 3.5; Sabourauds Dextrose Agar (SDA), pH 5.6; Starch Casein Nitrate agar (SCN), pH 7.2 and Malt Extract Agar (MEA), pH 5.5.

Production and extraction of the fungal pigment

The inoculums of the isolated fungus were grown in 250 ml Erlenmeyer flasks containing 100 ml of SCN broth. The non-diffusible red pigment produced by the organism was extracted by solvent extraction method using ethyl acetate. Mass production of the pigment was performed using submerged culture using SCN media. The fermentation broth was incubated at 30°C for 7 days. After successful culturing, the mycelia were revived by filtration from the fermented broth and were subject to ethyl acetate extraction. The extracted pigment in ethyl acetate was subject to rota vacuum evaporation and the crude pigment was evaporated and stored for further use.

Characterization of crude pigment: TLC, HPTLC and UV Visible spectrophotometry

Thin layer chromatogram of crude pigment was performed on pre coated silica gel 60 F_{254} plates using toluene: ethyl acetate (93:7) solvent system. High performance thin layer chromatography (HPTLC) analysis was carried out on a HPTLC (Camag, Switzerland) system with the same solvents. The crude pigment was subjected to UV Visible spectrophotometry in the entire scan range of 1100-199 nm using UV visible spectrophotometer 1800, Schimadzu made in Japan.

Assessment of the anthelmintic activity

Egg hatch assay

Fresh ova were collected from fecal sample of a domesticated goat infested with *Haemonchus contortus* and were concentrated by centrifugation. Eggs were washed with distilled water prior to the experiment. The crude pigment from *Aspergillus terreus* was used for the study. Albendazole and Ivermectin were used as positive control whereas 5% DMSO served as negative control. The extracts were diluted to concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml in a total volume of 0.5 ml. In the experiment, about 50 eggs/0.5 ml distilled water were counted and taken in marked 6- well tissue culture plates and were added with 0.5 ml of the extract dissolved in 5% DMSO. The effective concentration of the drug was thus reduced to 50, 25, 12.5, 6.25, 3.125 and 1.5625 mg/ml. Albendazole was also diluted using DMSO to provide concentration of 1 and 0. 5 mg/ml. The culture plates were incubated for 48 hrs at 28°C. The experiment was done in triplicates for each concentration. Hatched larvae (dead or alive) and unhatched eggs were counted under dissection microscope (magnification 40 X) [16, 17, and 18].

Assessment of the larvicidal activity

Five grams of dung from goats infested with *Haemonchus contortus* were incubated at room temperature with adequate humidity in dark for 10 days to get L3 larvae. The larvae were washed out into petriplates. The larvicidal activity was done as per the procedure of Rahman *et al.,* 2011 [19] with minor modifications. Approximately 25 motile larvae were collected in 0.5 ml water into which equal quantity of extract diluted in 5% DMSO was added. The extracts were diluted to concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml in a total volume of 0.5 ml. The effective concentration of the sample is thus diluted to 50, 25, 12.5, 6.25, 3.125 and 1.5625 mg/ml respectively. Albendazole and Ivermectin were used as positive control where as 5% DMSO served as negative control. The loss of motility of the larvae was checked every 15 minutes and the % larvae found non-motile/ dead were calculated.

Determination of antibacterial activity

The antimicrobial activity of the pigment dissolved in DMSO was tested by agar well diffusion method [20]. The activity was tested against MTCC strains of various human and animal pathogens namely *Escherichia coli* (40), *Salmonella typhimurium* (3224), *Pasteurella multocida* (1148), *Pseudomonas aeruginosa* (4999), *Staphylococcus aureus* (3160), *Streptococcus pyogenes* (1928), *Listeria monocytogenes* (657), *Enterococcus fecalis* (9845) and *Klebsiella pneumonia* (7028). For the preparation of inoculum, a suspension of the culture was transferred into a sterile tube and the turbidity was measured to Mcfarland Standard 0.5, corrected by using sterile normal saline. Sterile MHA plates were prepared and the bacterial cultures were swab cultured on to the surface of the agar. Wells of 6 mm diameter were bored using a sterile well borer and samples were prepared in various concentrations ranging from 100-2500 µg/ml of the extract diluted in 10% dimethyl

sulphoxide. 2500 µg/ml of chloramphenicol and 10% DMSO were used as positive and negative control. The plates were incubated at 37 °C for 24h after which the activity was evidenced by the presence of zone of inhibition surrounding the well. The experiment was done in triplicates, zone of inhibition measured, and the antibacterial activity was expressed as the mean diameter of zone inhibition (mm).

Minimum inhibitory concentration

The minimum inhibitory concentration of the crude pigment from *A. terreus* was performed by serial microplate dilution method of Eloff, 1998 [21]. The method was slightly modified by using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) instead of p-iodonitrotetrazolium violet INT. This method allows the determination of the minimum inhibitory concentration (MIC) of crude extract against each bacterial species by measuring reduction of tetrazolium violet. The bacterial cultures were incubated in Mueller-Hinton (MH) broth overnight at 37°C and a 1% dilution of each culture in fresh MH broth was prepared prior to use in the micro dilution assay. 100 µl each of bacterial culture and the pigment sample were added to 96 well microtitre plates. The plates were incubated overnight at 37 °C and bacterial growth was detected by adding 40 µL MTT (5 mg/ml) (Sigma) to each well. After incubation at 37°C for 1 h, MTT is reduced to a purple formazan by biologically active organisms, in this case, the dividing bacteria. The well in which the solution remained clear was shown to inhibit the growth of bacteria. This concentration was taken to be the minimum inhibitory concentration (MIC). Sterile broth containing extract alone and the standard antibiotic chloramphenicol (Sigma) were included in each experiment as negative and positive controls.

RESULTS AND DISCUSSION

Isolation and characterization of *Aspergillus terreus*

A red pigmented fungal culture was isolated and purified on SCN media. The red pigmentation was found to be prominent only in SCN medium which may be due to the neutral pH of the medium or the presence of casein or other minerals in the original media whereas it produced pale yellow color in PDA and yellowish orange color in SDA and MA. (Fig. 1). The macroscopic appearance of the colonies on SCN showed white, cottony and fluffy spores with reverse side intracellular orangish - red appearance (Fig. 2).

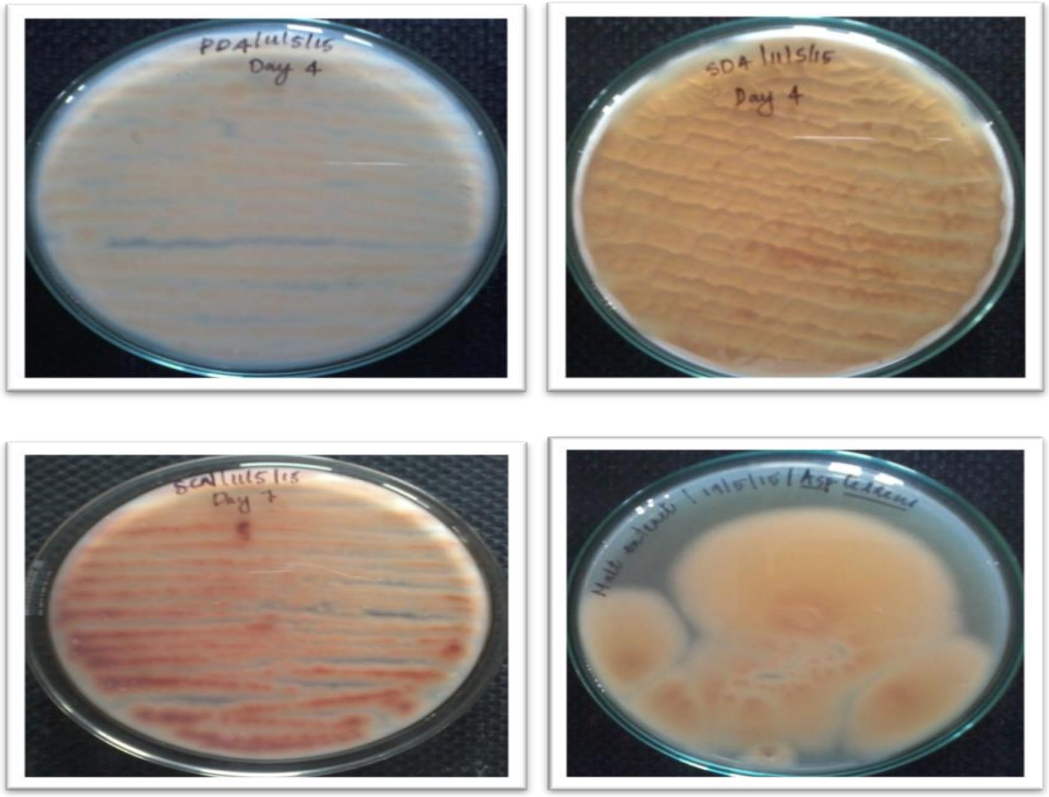

Fig. 1 Pigment production on different medium (PDA, SDA, SCN and MA)

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Fig.2 Growth of *A. terreus* on SCN media

The microscopic characterization of the organism using slide culture method showed biseriate structure containing septate hyphae and numerous phialide type conidia with straight unbranched conidiophores. The top of the conidiophores bulged to become vesicles with metulae and phialide. The molecular characterization was done using partial sequencing of 5.8s rRNA and the blast search analysis confirmed that the isolated organism was *Aspergillus terreus.*

Aspergillus is one of the oldest genera of fungi described by Micheli in 1729 [22]. *Studies of Eszter et al., 2011 [23] established that Aspergillus terreus* produces two types of asexual conidia: accessory conidia (AC, asexual conidia formed directly on hyphae) and phialidic conidia (PC, asexual conidia arising from conidiophores generated on hyphae). Accessory conidia are produced both *in vitro* and *in vivo*, and a recent study indicated that AC were morphologically distinct from PC in that they lacked a pigment-like outer layer and were larger than PC. They demonstrated other phenotypic differences between these two conidial forms including the ability of AC to germinate more rapidly, enhanced adherence of AC to microspheres, heightened AC metabolic activity, less cell membrane ergosterol and lower susceptibility of AC to the antifungal drug amphotericin B.

Extraction and pigment production

The pigment from *A. terreus* was extracted using ethyl acetate as the solvent. The organism was mass cultured by submerged culture method using SCN broth (Fig. 3.). A variety of extraction schemes have been reported for removal of pigments from cells. The methods used are empirical and usually involve extraction with single solvent or solvent mixtures [24]. The diversity of methods required to remove pigments from whole cells may reflect differences in solubility or differences in location.

Fig. 3 Culture after submerged fermentation

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Characterization of the pigment: Thin layer chromatography, HPTLC and UV Visible spectrophotometry

Thin layer chromatogram of the crude pigment revealed three distinct spots in the fluorescent region with *Rf* of 0.05, 0.62 and 0.81. HPTLC of the crude pigment revealed the presence of at least 12 different polyvalent compounds in the fluorescent range (Fig. 4). The maximum peak area of 32.62% was obtained for compound 2. The UV Visible spectrum of the crude pigment showed multiple absorption peaks between 200 and 400 nm. Absorption of 3.157, 3.967 and 3.189 was observed at 224, 264 and 352 nm respectively (Fig. 5).

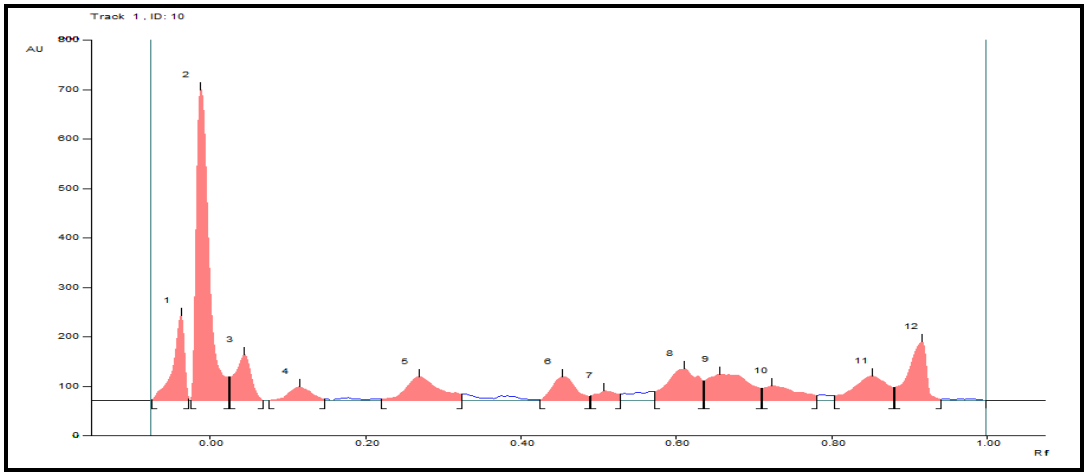

Fig. 4 HPTLC graph of crude pigment

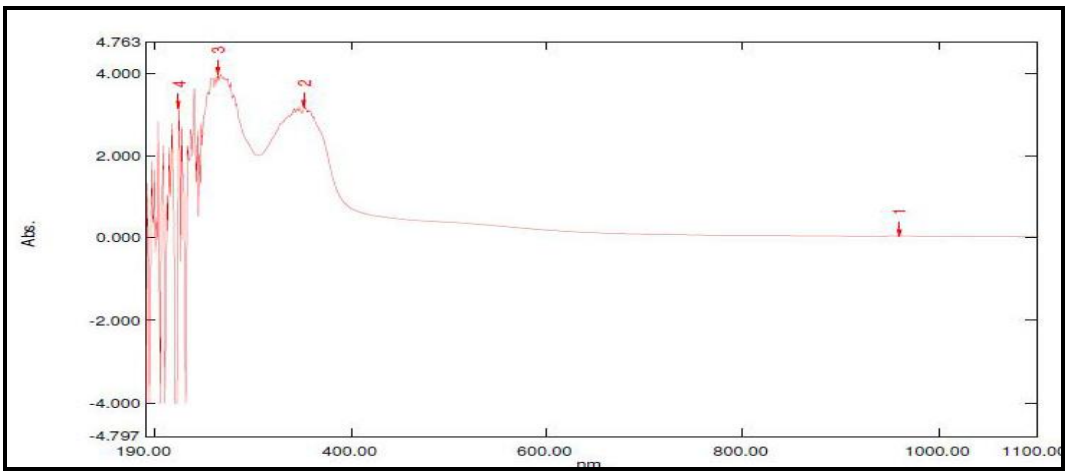

Fig. 5 UV Visible spectrum of crude pigment

Assessment of anthelmintic activity

Egg hatch assay

The hatching inhibition of *H. contortus* eggs by the crude pigment extract was observed in a dose dependent manner. In the highest concentration of the pigment i.e. at 50 mg/ml the morulla stage ova was found to be evacuated of its contents. In the second highest concentration, 50% of the ova had hatched to L1 stage and were dead and the remaining 50 were in its morulla stage. In the subsequent three concentrations the ova was in its dead L1 stage. In the lowest concentration of the drug the ova had hatched to its developmental L2 stage, where the larva comes out of the shell and was found to be dead in the presence of the pigment. In the negative control all the eggs had hatched to its developmental L2 stage and were found to be actively motile (Table 1). Albendazole and Ivermectin at both doses produced 100% death. Albendazole and ivermectin showed disintegration of ova in all the doses selected for the study. There was destruction of the entire number of ova counted.

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Table 1. Percentage of *Haemonchus contortus* eggs found dead after treatment with crude pigment

S. No.	No. of ova	Concentration of pigment (mg/ml)	Number of dead ova (%)
1	100	50	100 (Dead Morulla stage)
2	100	25	81(Dead Morulla and L1 stage)
3	100	12.5	77(Dead L1 stage)
4	100	6.25	65(Dead L1 stage)
5	100	3.125	59(Dead L1 stage)
6	100	1.5625	32(Dead L2 stage)

Larvicidal activity

The crude pigment was found to produce mortality in the L3 stage larvae of *H. contortus*. Maximum activity was seen within 60 min at which the pigment killed L3 larvae at the concentration of 50 mg/ml. The least activity was produced at 150 min at a concentration of 1.5625 mg/ml of the pigment (Table. 2). Albendazole and ivermectin at both concentrations produced 100% mortality within the first observation.

Table 2. Effect of the crude pigment extract on larval motility cessation

S. No.	No. of larvae	Concentration of the pigment (mg/ml)	Time taken for cessation of larval motility (min)
1	25	50	60
2	25	25	75
3	25	12.5	90
4	25	6.25	105
5	25	3.125	120
6	25	1.5625	150

A large number of medicinal plants have been used for treatment of parasitic diseases in man and animals. Similarly compounds of natural origin such as microbial pigments can also be screened for its various biological activities including anthelmintic activity. The genus *Aspergillus* encompasses organisms whose characteristics are of high pathological, agricultural, industrial, pharmaceutical, scientific and cultural importance and play an important role in the degradation of organic substrate [25, 26, and 27]. In the current study the pigment from *Aspergillus terreus* have been used to evaluate its anthelmintic potential. Screening of anthelmintic activity is mainly through *invitro* tests including larval and adult paralysis/ death, egg hatch assays or motility and biochemical tests [28]. *Invitro* tests using the larvae of *Haemonchus contortus* is considered to be one of the best means of screening drugs for anthelmintic activity [29].

The pigment possessed good anthelmintic activity against ova of *Haemonchus contortus*. The larvicidal activity of the pigment was not appreciable as that of the ovicidal activity by the pigment even though the pigment brought about the cessation of motility of the larvae even at the lowest concentration of the sample tested. The action on the cell membrane of the ova was very evident from the evacuation and disintegration of the ova at higher doses of the pigment treated groups. In the case of larvae, there was reduced motility from the initiation of the experiment itself which could be due to the effects of the extract on the energy metabolism of the parasite [30].

Antibacterial activity and MIC

Among various organisms tested good antimicrobial activity was seen against both gram positive and gram negative organisms in the concentration range of 100-200 µg/ml. The MIC of the sample against susceptible organisms was also determined using tetrazolium compounds. The results are tabulated in Table. 3.

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Table 3. Antibacterial activity and MIC

S. No	Name of the organism (MTCC no)	Zone of inhibition (mm) Concentration of the pigment (µg/ml)							MIC (µg/ml)
		100	200	300	400	500	1000	Chloramphenicol (2.5 mg/ml)	
1	*Klebsiella pneumoniae* (7028)	10	12	14	15	16	20	20	60
2	*Salmonella typhimurium* (3224)	15	18	21	22	24	26	33	60
3	*Escherichia coli* (40)	15	17	18	20	23	26	30	60
4	*Listeria monocytogenes* (657)	-	-	16	18	25	28	30	240
5	*Staphylococcus aureus* (3160)	10	12	15	21	29	30	18	70
6	*Streptococcus pyogenes* (1928)	-	11	18	19	24	26	21	80
7	*Pasteurella multocida* (1148)	-	-	-	-	-	10	20	600

Potent activity of the pigment was shown against *Klebsiella pneumoniae* with an inhibition zone of 10 mm and against *Escherichia coli* and *Salmonella typhimurium* with 15 mm of inhibition at 100 µg/ ml of sample. The MIC values for the organisms determined using micro dilution assay was also found to be 60 µg/ ml for *K pneumoniae*, *E coli* and *S typhimurium*. In the case of *Staphylococcus aureus* and *Streptococcus pyogenes* the minimum inhibitory zones was produced at 100 and 200 µg/ ml of sample with zones of 10 and 11 mm respectively and the MIC was found to be 70 and 80 µg/ ml. Thus the sample was found to have a wider inhibitory effect against gram negative organisms. The Gram-negative bacteria are much more complex and less permeable to drugs and they show variations in inhibition against antibiotics. These properties are generally attributed to the cell wall composition differences of gram positive and gram negative organisms [31]. The gram negative bacteria contribute to almost all major infections in humans and are most difficult to treat by conventional antibiotics. The significant inhibition of the Gram-negative bacteria is therefore of great importance, due to their resistance to antibiotics [32]. As such, the number of compounds isolated from micro organisms is increasing faster when compared with other sources [33].

CONCLUSION

The results of the present study highlight that the intracellular red pigments from *Aspergillus terreus* possess efficient antibacterial activity as well as anthelmintic potential. The future work is aimed at purification, characterization and analyzing the biological activity of the pigment.

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