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Effect of *Pottia lanceolata* Linnaeus (Moss Extract) on *Trichostrongylus* in Sheep

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Abstract:
Bryophyte species, *Pottia lanceolata* was used as anthelmintic, against sheep nematode *Trichostrongylus in vitro.* Aqueous extract of *Pottia lanceolata* exhibited anthelmintic activity in dose dependent manner showing maximum efficacy at 20 mg/ml concentration. To test the efficacy of the treatment transcriptome analysis was done after isolating and extracting the mRNA. Gene expression pattern were studied in both the control and treated samples. A total of 1,763 genes were expressed in the control and treated samples, the genes expressed in control and treated samples differed. It was found that certain genes were up regulated in the control (257) and certain genes were down regulated (341). The transcriptome data as mRNA sequences were submitted to public database, GEO. The following are the ids of the submitted sequences. (GSE137609) Sheep Nematode Control (GSM4083097) Sheep Nematode treated (GSM4083098). The treatment was found to be quite effective, as expression of unique genes was observed, which seem to play a major role in apoptosis, cuticle damage, neuronal destruction, and triggering major signaling pathways, causing obliteration of worms. The bryophyte extract seems to be effective in inhibiting the nematode activity by impacting the genome and gradually leading to the extermination of the parasite and therefore may be suggested to be used as an effective nutraceutical for this helminth infection.

Keywords: Moss extract, *Pottia lanceolata, Trichostrongyle,* Anthelmintic, mRNA, Ttranscriptome analysis, GEO, Nutraceutical, Sheep Nematode

INTRODUCTION

It is a common practice, in animal farming, that animals, are often treated with chemical anthelmintics to rid of their infections. There is repeated use, of these drugs, without any regulations, which seem to be ineffective in controlling the helminth parasitic infections. Constant and indiscriminate use of synthetic anthelmintics, might lead to resistance as reported in certain nematodes, of sheep and goat (Kebede, A., 2019). In this context, a more amicable solution, as developing efficient measures, to prevent, control and mange the infections would yield better results as suggested by Maurer *et al. (*2007). There are instances, from various parts of the world, where in drug resistance has been reported against anthelmintics (Maingi *et. al.,* 1998, Paraud *et. al*., 2009**)**. Certain states in India, also have reported anthelmintic drug resistance in sheep and goat from animal farms.(Dhanalakshmi *et. al*., 2003; Jeyathilakan *et. al*., 2003; Deepa and Devada, 2007; Easwaran *et. al.,* 2009). From these studies,

it is quite evident that constant and indiscriminate use of synthetic anthelmentic drugs though might be effective for brief periods, of time; in due course there is a threat of developing resistance. Therefore there is often, a constant search for developing better alternatives to synthetic drugs and in this regard, plants and their secondary metabolites have shown promising results as effective therapeutic agents. Plant extracts have long been used to treat various helminth parasites of animals and humans. Paralytic effect of *Allium sativum* and *Piper longum* upon liver amphistome of the species *Gigantocotyle explanatum* was reported by Singh, *et al.*, (2008). Apart from these plant extracts, certain other natural extracts such as algae, bryophytes and pteridophytes are also known to treat various pathogens. Antimicrobial activity of epiphytic moss *Stereophyllum ligulatum* was studied by Chaudhary and Prem Kumar (2011). Anti microbial, anti cancerous and antioxidant effects of mosses have also been reported, by several authors, (Vanhoof, *et al.,* 1981, Gunatilakaa, 1994) but anthelmintic activity of bryophytes has so far not been reported. Our initial studies, using bryophyte extract of the species, *Pottia lanceolata*, had shown promising results, in elimination of chick helminths *in vitro* (Sunila Kumari, 2016). As these results, were quite encouraging, it was intended to further study the effect of bryophyte on sheep nematodes, at the transcriptome, level. Certain times, there may be possibilities, that these naturally occurring compounds, may be exploiting distinctive pathways, or strategies, that might be different from the presently exploited targets, of synthetic anthelmintics drugs (Hrckova, *et al.,* 2013; Roeber F, Kahn L, 2014) and so may be successful as anthelmintic nutraceutical. Therefore we hypothesize that the bryophyte extract can be effective in inhibiting the nematode activity by impacting the genome and gradually leading to the extermination of the parasite**.** The present study is an attempt to analyze the anthelminthic activity of the bryophyte extract, by analyzing the transcriptome of the parasite before and after treatment.

MATERIALS AND METHODS

Plant material collection and extraction

The bryophyte, *Pottia lanceolata was* collected from Osmania University College for women, Koti, Hyderabad and Golconda fort Hyderabad, Telangana, India. The plant material was taxonomically identified by the taxonomists of Botanical Survey of India, Hyderabad. A voucher specimen has been preserved in our laboratory for future reference. The plant material was dried in shade, pulverized, passed through sieve number 40 and stored in air tight container and used for further extraction.

Preparations of Aqueous extract (Maceration method)

Bryophyte, *Pottia lanceolata* samples were cleaned removing soil debris, by thorough washings with water. The active ingredient was extracted, using cold maceration method dried in shade, powdered using mortar and pestle. The crude aqueous extract was centrifuged, at 5,000 rpm for 5 minutes, using Remi centrifuge. The clear supernatant was used for the treatment and the precipitate was discarded.

Collection of parasites (Nematodes of Sheep)

For the present study, the desired parasites are Nematode parasites of sheep, *Trychostrongylus,* collected from the intestines of sheep. These parasitic worms were used to test the anthelmintic activity with bryophyte extracts *in vitro*. The identification of nematodes was done in the department of Zoology, OUCW, Koti, Hyderabad, INDIA, by using Phase contrast microscope.

Piperazine citrate (glaxo smithkline) was used as the standard anthelmintic drug during the experimentation.

Anthelmintic activity of Bryophyte extract

Anthelmintic activity assay was carried out as per the method of Ajaiyeoba *et al.,* (2001). To study the gene expression patterns, the control and treated (bryophyte extract, *Pottia lanceolata)* parasites were preserved in RNA later solution, and were subjected to RNA extraction and isolation using RNA mini easy kit. The Extraction was performed as per the protocol, which is discussed below.

RNA Extraction Protocol

Take the appropriate tissue (nematodes) present in RNA Later. Disrupt the tissue in liquid nitrogen as a powder and then add lysis buffer to the powdered tissue. Grind until homogenize completely.

Centrifuge the homogenized lysate at 10000 rpm for 5 minutes at 4°C. Pass through the supernatant in gDNA columns. Add 1 volume (usually 350ul or 600ul) of 70% ethanol to the flow through and mix well by pipetting. Transfer up to 700ul of the sample, including any precipitate, to an RNeasy spin column placed in a 2ml collection tube. Close the lid, and centrifuge for 15 s at ≥ 8000 x g. Discard the flow through. Precede the same step with left over sample and discard the flow through. Add 700ul Buffer RW1 to the RNeasy mini spin column (in a 2ml collection tube). Close the lid, and centrifuge for 15 s at ≥ 8000 x g. Discard the flow through. Add 500ul Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 Sec at ≥ 8000 x g. Add 500ul Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2min at ≥ 8000 x g. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30-50 ul RNase-free water directly to the spin column membrane. Close the lid and centrifuge for 1min at ≥ 200817 x g to elute the RNA. Store the eluted RNA in -80°c.s

The steps followed to generate the FastQ reads from Total RNA libraries:-
1. The pooled / multiplexed libraries were put into the Illumina Hiseq Sequencer for sequencing which generated raw data in the bcl format.
2. Bcl files were De-multiplexed using sample wise Barcode information to get the sample specific sequences.
3. De-multiplexing resulted into raw reads in FastQ format, which was used for downstream analysis. In this regard, the mRNA was extracted and isolated and further analyzed.

RESULTS

Anthelmintic activity assay was carried out as per the method of Ajaiyeoba *et. al*., (2001). The assay was performed *in vitro,* using nematodes of sheep. Test samples of the extract were prepared at the concentrations, 10, 20, 50 and 100 mg / ml of *Pottia lanceolata* bryophyte extract dissolved in distilled water. Six worms, each of sheep nematodes *Trichostrongylus* were placed in petridish measuring 9 cm in diameter. 25 ml of bryophyte extract was added to the petridishes. Piperazine citrate of concentration 10mg / ml was prepared and used as reference standard and double distilled water was used as control. Extract solution and standard solution were freshly prepared, prior to experimentation. Observations were made and the time taken for paralysis was noted when no movement of any sort could be observed. Time of death of worms were recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water. It was observed that at higher concentrations of bryophyte extract *Pottia lanceolata* produced paralytic effect much earlier and the time taken for death was shorter. Aqueous extract of bryophyte *Pottia lanceolata* exhibited anthelmintic activity in dose dependent manner showing maximum efficacy at 20 mg / ml concentration. Anthelmintic activity of the extract was compared with standard drug piperazine citrate. These results suggest that bryophyte extract of *Pottia lanceolata* exhibited significant anthelmintic activity. (Table 1)

Table 1: Anthelmintic activity

S. No.	Groups	Concentration Mg/ml	*Trichostrongylus species*	
			Time taken for paralysis in minutes (Mean and SEM)	**Time taken for death in minutes (Mean and SEM)**
1	Control Distilled water treated			
2	Bryophyte treated	10	30±1.30	45±1.62
		20	11±1.81	34±1.20
		50	10±1.70	30±1.00
		100	08±0.42	18±1.30
3	Standard Piparazine citrate	10	11±1.1 2	35±1.0

Analysis Report for Transcriptome Data
NGS Experiment Overview

Organism	: Sheep nematodes
Sequence length	: 150 nt
Adapter	: NEB Adapters
Protocol	: Illumina Transcriptome Library preparation
NGS Platform used	: Illumina NextSeq 500
Library layout	: Paired End
Sample IDs	: Sheep Nematode Control & Sheep Nematode Treated

***De-novo* Assembly**

All processed reads were assembled into transcripts without any reference (De-novo) both samples independently using Trinity software (trinityrnaseq-r20140413p1). It represents a novel method for the efficient and robust *de novo* reconstruction of transcriptomes from RNA-seq data.

UNI-Gene generation

Unigenes were generated after clustering the merged sequences from both Control and Treated samples using CD-HIT. It takes a FASTA format sequence database as input and gives a cluster file as output. The idea is to reduce the overall size of the database without removing any sequence information by only removing redundant sequences. Unigene is the master control data set which represents common sequences along with unique sequences from both the samples. Sheep Unigene contains a total of 93951 sequences.

Protein Annotation: Homology search was done for Unigene sequences against Nematodes proteins downloaded from Uniprot database (http://www.uniprot.org/). ncbi-blast 2.2.29 was used for homology search between the sequences.

Differential gene expression: The reads for the samples (Sheep) were separately aligned to the corresponding unigene sequences and read count profiles were generated. DESeq "R" package was used for differential gene expression. The package DESeq provides methods to test for differential expression by use of the negative binomial distribution and a shrinkage estimator for the distribution's variance. Transcripts were classified as expressed in both the samples, or only present in either of the samples (expressed only in control sample or expressed only in treated sample**).** Gene expression pattern was studied in both the control and treated samples. A total of 1,763 genes were expressed in the control and treated samples, the genes expressed in control and treated samples differed. It was found that certain genes were up regulated in the control (257) and certain genes were down regulated (341) (Table 2). In the treated samples, it was found that there was expression of certain unique genes. The transcriptome data as mRNA sequences were submitted to public database, GEO Submission (GSE137609). Sheep Nematode Control (GSM4083097), Sheep Nematode Treated (GSM4083098).

Table 2: DGE Sheep Control and Sheep Treated- Statistics

Category	Total	Up	Down	Neutral
Both Control and Treated	43810	15301	17132	11377
Both Sheep Control and Sheep Treated	32433	15301	17132	0
Only Sheep Control	20725	NA	NA	NA
Only Sheep Treated	38598	NA	NA	NA

Transcriptome data of mRNA was analyzed by constructing the De novo assembly using trinity software.

Based upon these results, a huge database of protein expression has been generated, but few unique protein which were expressed in treated samples which played an important role in mortality of worms have been discussed in the following section. (Table 3)

Table 3: Important role in mortality of worms

S. No.	Protein	Differentially Expressed	Control	Treated
1	Asparagine synthetase a	NO	Absent	Present
2	Beta-lactamase (Fragment)	NO	Absent	Present
3	Catalase	NO	Absent	Present
4	Cathepsin L-like cysteine proteinase	NO	Absent	Present
5	Degenerin deg-1 (Degeneration of certain neurons protein 1)	NO	Absent	Present
6	Leishmanolysin	NO	Absent	Present
7	UNC-63	NO	Absent	Present

DISCUSSION

In the present study, as hypothesized, bryophyte extract prepared from the species, *Pottia, lanceolata*, was found to be effective in eliminating the *Trichostrongylus* parasite *in vitro*. This may be attributed to expression of certain unique genes and their proteins, which may have triggered certain signaling pathways, leading to extermination. Quite a number of studies, illustrating various medicinal properties of mosses, such as antibacterial, antifungal antitumor properties have been reported by several authors (Satish Chandra *et al*., 2017). In addition to these properties, a recent study on bryophytes, has revealed the presence of terpenoid and aromatic compounds, which are known to have neuro protective effect on nervous system of humans (Agnieszka Ludwiczuk and Yoshinori Asakawa, 2020). However, anthelminthic property of bryophytes has so far not been explored or reported. Present study is the first of its kind, where efficacy of bryophytes as potential anthelminthic agents, was studied at transcriptome level. In the present study, sheep parasites were treated with different concentrations, of the extract, and this treatment was found to be effective in eliminating the parasite. To ascertain, the mechanism of action of the extract, transcriptome, analysis was done, by extracting and isolating the mRNA, and later subjecting it to gene sequencing using illumina nextgen sequencer. The analysis revealed expression of certain proteins exclusively in the treated samples, suggesting, that bryophyte regimen, had influenced their expression which may have impacted the survival of the worms and led to their elimination. As the data generated from this analysis is quite humongous, a few important proteins have been briefly discussed as follows. There was expression of Asparagine synthetases a. in the treated samples, probably to aid the survival of the parasite in wake of the treatment, as this enzyme is known to be an important and essential enzyme, for the survival of parasites as seen in case of *L. donavani* (Faria, J. *et al*, 2016). Similarly β Lactamase enzyme was also expressed in the drug treated samples and this seems to be a defense strategy by the nematodes, to counter the β lactums found in moss extract, as bryophytes are known to contain plethora of bacterial biota. (Faisal Hammad *et al*., 2015). This conclusion is similar to the observation found in gram negative bacteria which show the presence of this enzyme, against β lactam antibiotics,(Nichols *et al,* 2014)[21]. In addition to these two enzymes, quite a number of other proteins have been expressed under the influence of the extract. It is interesting to note that there was expression of two sets of diversely antagonistic proteins, in response to the treatment. On one hand, enzymes, such as catalase, and protein like leishmanolysin, were synthesized, to rescue the nematode from adverse effects of moss as these molecules are known to be critical for the survival of the parasite (Harris *et al*, 2002; Joshi, 2002). But on the other hand, destructive proteins, like degenerin Deg1 (degeneration of certain neuron protein 1), and cathepsin L. Like cysteine proteinase (Cath L) were also expressed. Deg1 protein is known to form channels, and cause neuronal degeneration by causing cell vacuolation, cell swelling and death (Jaime, Gracia-Anoveros *et al*, 1995). Enzyme, Cathepsin L. Like cysteine proteinase (Cath L) acts as an insecticidal and is known to cause immune related proteolytic activation cascade leading to production of active phenol oxidase (Pyati, P.S *et al,* 2009) which may

cause damage to the cells and death of the nematode. Apart from these, there is also synthesis of a unique protein, known as UNC 63. This gene encodes for nicotinic acetylcholine receptor α subunit in *C. elegans* (Culetto *et. al.*, 2004) and is known to cause resistance to anthelmintic drug levamisole. Expression of this protein in the present study, suggests that, *pottia lanceolata* extract may seems to contain a natural compound which may be similar to synthetic anthelmintic drug levamisole, which may have triggered the activation of UNC63. From these observations, it may be concluded that bryophyte treatment was quite effective, and under the influence of the treatment, there was expression of two sets of diverse proteins, which on one hand, were expressed for survival of the worm, in response to stressful condition and on the other hand, the other set triggered expression of certain proteins, which may have elicited a cascade of signaling pathways, which seem to have devastating effect upon the nematode parasite leading to its mortality. In summary, it may be said that there was activation of both life saving and life threatening gene expression, but the expression of destructive mechanisms as apoptosis, channel formation and other oxidation pathways seems to have overpowered all the rescue machinery of the parasite, ultimately causing the destruction of the *Trichostrongylus* parasite. This study distinctly, demonstrates, the occurrence of certain natural anthelmintic compounds of *Pottia lanceolata*, and so may be recommended as a potent anthelmintic nutraceutical.

CONCLUSION

In conclusion, it may be said that *Pottia lanceolata* treatment was found to be quite effective as an anthelmintic agent against *Trichostrongylus*. The treatment, initiated expression of unique genes, which evoked several antagonistic pathways, leading to apoptosis, cuticle and cell damage, and also causing neuronal destruction leading to obliteration of the nematode. Further studies, may be undertaken, so as to identify and extract and isolate the magic anthelmintic compound, which may be further processed and tested in *vivo* and advocated as a dynamic nutraceutical for commercial application.

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Conflicts of Interest

Authors declare that there is no conflict of interest

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