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EFFECT OF BRYOPHYTE, *POTTIA LANCEOLATA* AS NATURAL ANTHELMINTIC UPON NEMATODE PARASITE *ASCARIDIA GALLI*

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ABSTRACT : Helminth infestations of poultry are a major concern, as they are known to impact the livestock farming in a big way. There is often, use of synthetic or chemical anthelmintics, to control these infections, however these chemicals, seem to be effective only for a brief period of time, as they seems to lose their efficacy, because of the parasites, developing resistance against these drugs. In this context, it has often been debated, and suggested that naturally occurring compounds, of different plant taxa, may be quite effective as anthelmintics, and the parasites, seldom develop resistance. In lieu of these, observations, in the present study, aqueous extract of bryophyte, *Pottia lanceolata* was investigated for anthelmintic activity upon *Ascaridia galli in vitro.* The nematode parasites, were collected from naturally infected chicken and different dosages, of water extracts, were used and significant anthelmintic activity was observed at the concentration of 20 mg/ml. To know the effectiveness of this herbal drug at the genome, level, transcriptome analysis was done, by extracting isolating and sequecing of the mRNA, of treated and untreated samples, using Illumina nextgen sequencing (NGS). The sequencing data, as mRNA sequences, were submitted to public data base, GEO (Gene Expression Omnibus) from NCBI. (Chick nematode control is GSM4083095 and chick nematode treated is GSM4083096). Transcriptome analysis revealed, expression of certain unique genes, and also showed up regulation and down regulation of certain genes. The present study confirms that an effective natural anthelmintic could be developed using *Pottia lanceolata.*

Key words : Bryophyte, *Pottia lanceolata*, *Ascaridia galli*, anthelmintic, transcriptome, GEO.

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INTRODUCTION

Helminthosis is a disease of animals caused by gastro-intestinal helminthes, which seem to impact the profitable livestock business in many developing countries (Cheijina, 2001). Helminth parasites of poultry and sheep are usually controlled by repeated anthelmintic treatments of the flock. Constant use of chemical anthelmintics has resulted many a times, resistance to these drugs, thus making them ineffective (Maingi *et al*, 1998; Paraud *et al*, 2009). In this context, there are instances, where in alternate treatments for gastrointestinal helminthes in poultry and sheep have been investigated by several authors, like Lal *et al* (1976), Javed *et al* (1994), Singh and Nagaich (2002). However, significantly data on the efficacy of herbal treatments against adult and larvae of *Ascaridia galli, Heamonchus contortus*, *Railleitina tetragona* are quite limited. Literature survey reveals medicinal value, particularly anthelmintic effect of many plant extracts on 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). Even lower group of plants, 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). These studies suggest that bryophytes can be effective against certain pathogens and so we speculate that bryophytes can also be effective anthelminthic agents against helminthes of poultry. Of lately, it is interesting to learn about the adaptive and resistant strategies employed by these

parasites, to evade the synthetic drugs and is quite successful in survival and also to perpetuate the infection. It is found that in spite of use of many known chemical anthelminthics, there is no complete control or elimination of the nematode parasites, particularly *Ascaridia galli*. This nematode parasite is known to infect the poultry in a big way, after seemingly to be eliminated after a period of 10 years. This clearly indicates that the parasite seems to have adapted some new survival strategies by adjusting physiologically, or by may be developing certain new anatomical structures. Based upon these studies and observations, it is quite reasonable to assume that if a new plant based anthelminthic drug can be developed, which can effectively target the parasite genome, thereby reducing its ability to transform or inhibit the parasite from developing new mechanisms, or triggering some novel pathways, will be of great advantage. 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, belonging to the species, *Pottia lanceolata* by analyzing the transcriptome of *Ascaridia galli* 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 Poultry)

For the present study, the desired parasites are *Ascaridia galli* which parasitizes chicken. The intestines of freshly slaughtered fowls, were collected from local slaughterhouse and washed with normal saline solution. These intestines were then dissected and the nematodes were extracted. The average size of the nematode was found to be 5 cm. 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 by using phase contrast microscope.

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

Based upon these results, it was decided to study the gene expression studies. For this purpose, 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, provided by the manufacturer. Extracted RNA was subjected to quality and quantity check, prior to library construction. Library preparation was performed following NEB Next Ultra RNA Library Prep Kit (#E7490L). In brief, approximately 110-350 ng of Total RNA was used to isolate mRNA using Poly dT beads. mRNA was fragmented and converted to cDNA according to the protocol. cDNA was end repaired and purified using Ampure XP beads. Cleaned DNA was adapter ligated and purified using Ampure XP beads. These adapter ligated fragments were subjected to 12cycles of PCR using primers provided in the kit. The PCR products were purified using Ampure XP beads. Quantification and size distribution of the prepared library was determined using Qubit flourometer (Table 1) and Agilent Tape station D1000 Kit (Agilent Technologies) according to the manufacturer's instructions. The steps followed to generate the FastQ reads from Total RNA libraries are as follows:

The pooled/multiplexed libraries were put into the Illumina Hiseq Sequencer for sequencing which generated raw data in the bcl format. Bcl files were De-multiplexed using sample wise Barcode information to get the sample specific sequences. De-multiplexing resulted into raw reads in FastQ format, which was used for downstream analysis.

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 poultry. Test samples of the extract were prepared at the concentrations, 10, 20 and 50 mg/ml of *Pottia lanceolata* bryophyte extract dissolved in distilled water. Six worms, each of chicken nematodes *Ascaridia galli* 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 for 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 .The plant extract exhibited maximum efficacy 20mg/ml against *Ascaridia galli*. 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). As the extract had an effect upon, parasitic nematode, it was intended to study the gene expression pattern of chicken nematode parasite, *Ascaridia galli*. For these studies, the mRNA was extracted, isolated and further analyzed as per the protocol, described earlier. All the 150bp paired end raw reads (IlluminaNextSeq500) were quality checked for low quality bases and adapter sequences (Processing of raw reads). Quality check and processing was done using In-house perl scripts.

De-novo assembly

Table 1 : Anthelmintic activity of Bryophyte *Pottia lanceolata* aqueous extract.

S. No.	Groups	Concentration	*Ascaridia galli*	
		Mg/ml	Time taken for paralysis in minutes (mean and SEM)	Time taken for death in minutes (mean and SEM)
1	Control Distilled water			
2	Bryophyteextract	10	22±1.20	55±1.22
		20	14±1.82	45±1.40
		50	08±1.36	32±0.82
3	Standard Piparazine citrate	10	15±1.13	40±1.20

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.

Differential gene expression

The reads for the samples 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 and only present in either one of the samples (Please refer to the excel sheet at the end of the manuscript).

DISCUSSION

In the present study, bryophyte extract from the species, of *Pottia lanceolata* was found to be effective as anhelmintic. *In vitro* treatment, on the chicken nematode, *Ascaridia galli* showed mortality at 20mg/ml. dosage. As the mortality was observed, it was intended to learn about the, intricate details, which led to the death of the parasites, and hence transcriptome analysis was done. The transcriptome data has been submitted to public database, GEO (GSE137609) (GSM4083095 is chick nematode control and GSM4083096 is chick nematode treated). The transcriptome analysis has shown that approximately 2092 genes have been expressed. The gene expression pattern, of the control and bryophyte treated nematodes of chicken, showed a significant up regulation, down regulation of certain specific genes and also expression of certain unique genes. For convenient interpretation of transcriptome, mRNA data was translated to proteins, using trinity software, and some important proteins have been briefly discussed. Among the proteins, expressed, certain unique proteins, seems to have played a significant role either in the survival of the parasite, or which may have caused death, leading to extermination of the parasite. We observed, expression of the enzyme, Asparagine Synthetase in the bryophyte treated parasite samples. This is an important enzyme, known to be expressed and regulated under severe cellular stress as opined by Balasubramanian *et al* (2013). Production of this enzymes, during the treatment, regime, indicates that the bryophyte extract, seems, to cause, cellular stress, which may lead to apoptosis, and so the

expression of this enzyme. In addition to this, it is interesting to note that there was expression of a unique enzyme, bleomycin Hydrolase. This is a cytoplasmic cysteine peptidase, that is known to metabolically inactivate the anticancer drug, bleomycin (Rebecca *et al*, 2019). Expression of this protein in the treated samples, suggests that there may be release of a bleomycin like compound from the *pottia lanceaolata*, which may have a similar functionality as that of bleomycin *i.e.* DNA damage (Brandt Josiah and Valerie Gerriets, 2020) thereby causing the mortality of the worms and so to counter this compound, there seems to be the expression of this enzyme. Apart from these enzymes, there was also expression of proteins like, cuticle collagen DPY 7 fragment, Filamin A and filamin C and Glutathione reductase during the treatment regime. Cuticle collagen DPY 7 fragment is known to control the cuticle collagen formation and functionality (John *et al*, 1997), Filamin A and filamin C are the proteins related to cytoskeletal stability and anchorage (Christina De Maso *et al*, 2011) and Glutathione reductase, is an enzyme, which protects haemoglobin and biological cell membranes, against oxidative damage (Chang *et al*, 1978). Our observations, and understanding about the release of these proteins, under the influence of the treatment, suggests, that, the moss extract probably contains, a concoction of compounds, which seems to have a multi fold effect upon the parasite, so as to cause cuticular damage, disrupt the filamin integrity, and also causing damage to the membranes, which ultimately cause the death of the parasites, and so to salvage the parasite from the damage, there may be the expression of these proteins. These observations, suggest that moss extract prepared from *Pottia lanceolata* was found to be effective against the *Ascaridia galli* and so may be considered as an effective natural anthelmintic. In recent times, quite a number of medicinal properties of different species of moss have been explored and reported by many authors (Vanhoof *et al*, 1981; Gunatilakaa, 1994). However, anthelmintic properties have so far not be explored or tested. In the present study, *pottia lanceolata* was successful in eliminating helminth parasites, specifically *Ascaridia galli*, *in vitro* and therefore may be suggested as a natural anthelmintic drug. In summary it may be said, that natural extract of *Pottia lanceolata*, seems to contain certain specific compounds, which may be either directly involved in cuticle damage or membrane, destruction, or they seem to trigger certain specific signaling mechanisms, which may have deleterious effect upon the parasite, leading to its extermination. Further, the moss extract may be analyzed for the specific compound, which shows, anthelmintic activity may be isolated, tested *in vivo* and employed for commercial use.

CONCLUSION

In the present study, the bryophyte belonging to the species, *Pottia lanceolata* was tested for the efficacy of being used as the anthelmintic drug against the chicken nematode *Ascaridia galli*. The treatment was found to be quite effective, as the transcriptome analysis, revealed, expression of unique genes/proteins which seem to play a major role in the cuticle disintegration, DNA damage, and oxidative membrane damage and hence may be suggested that bryophyte extract may be effectively used as a natural anthelmintic to rid the chicken of helminth infection.

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